IPANEMA

Integration of PAper-based Nucleic acid testing mEthods into Microfluidic devices for improved biosensing Applications

PAPER-BASED MICROFLUIDICS AND MAGNETIC NANOPARTICLES FOR POINT-OF-CARE DEVICES. POTENTIAL SOLUTIONS FOR TOXIC CYANOBACTERIA DETECTION.

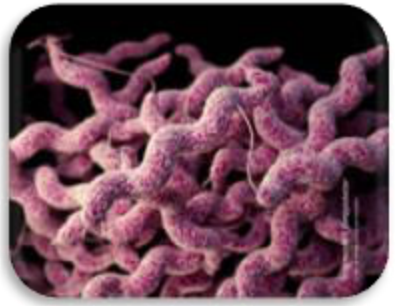
Jovana Stanojev, Ivana Podunavac, Nejra Omerovic, Petar Davidovic, Mila Djisalov

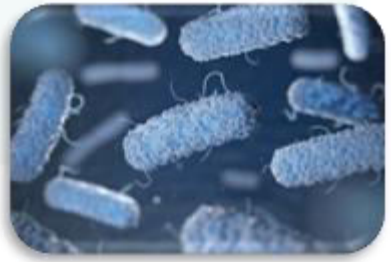
IPANEMA, OJC3, 03.06.2020.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics

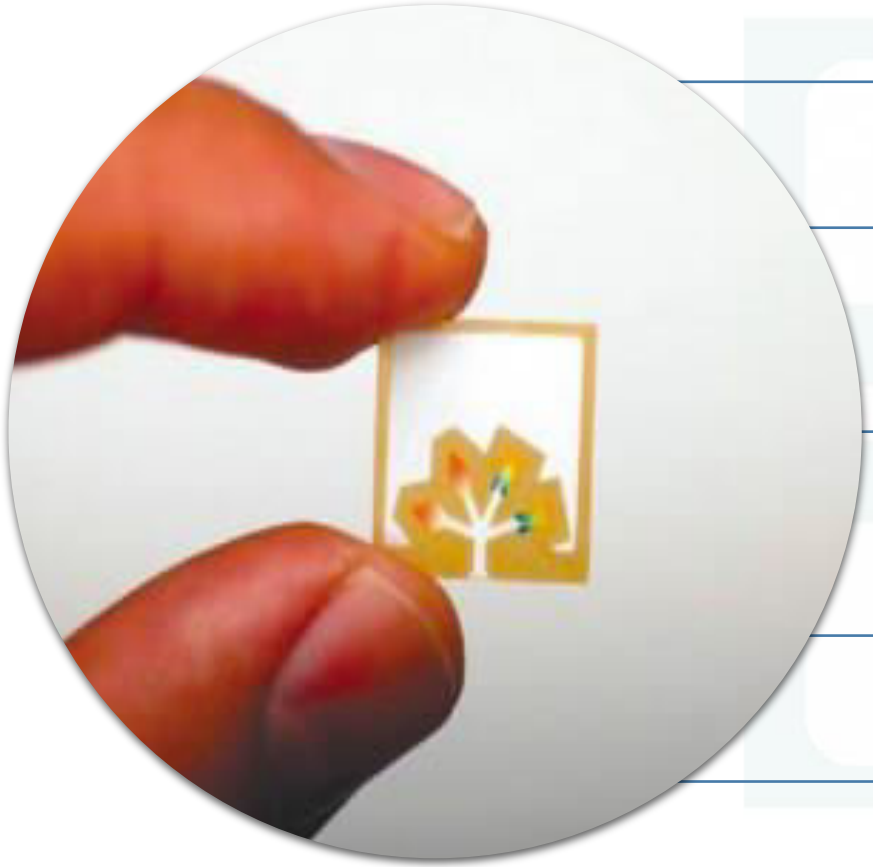
IPANEMA





This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics



- POC devices
- Affordable
- Accurate
- Versatile application
- No pumps

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics

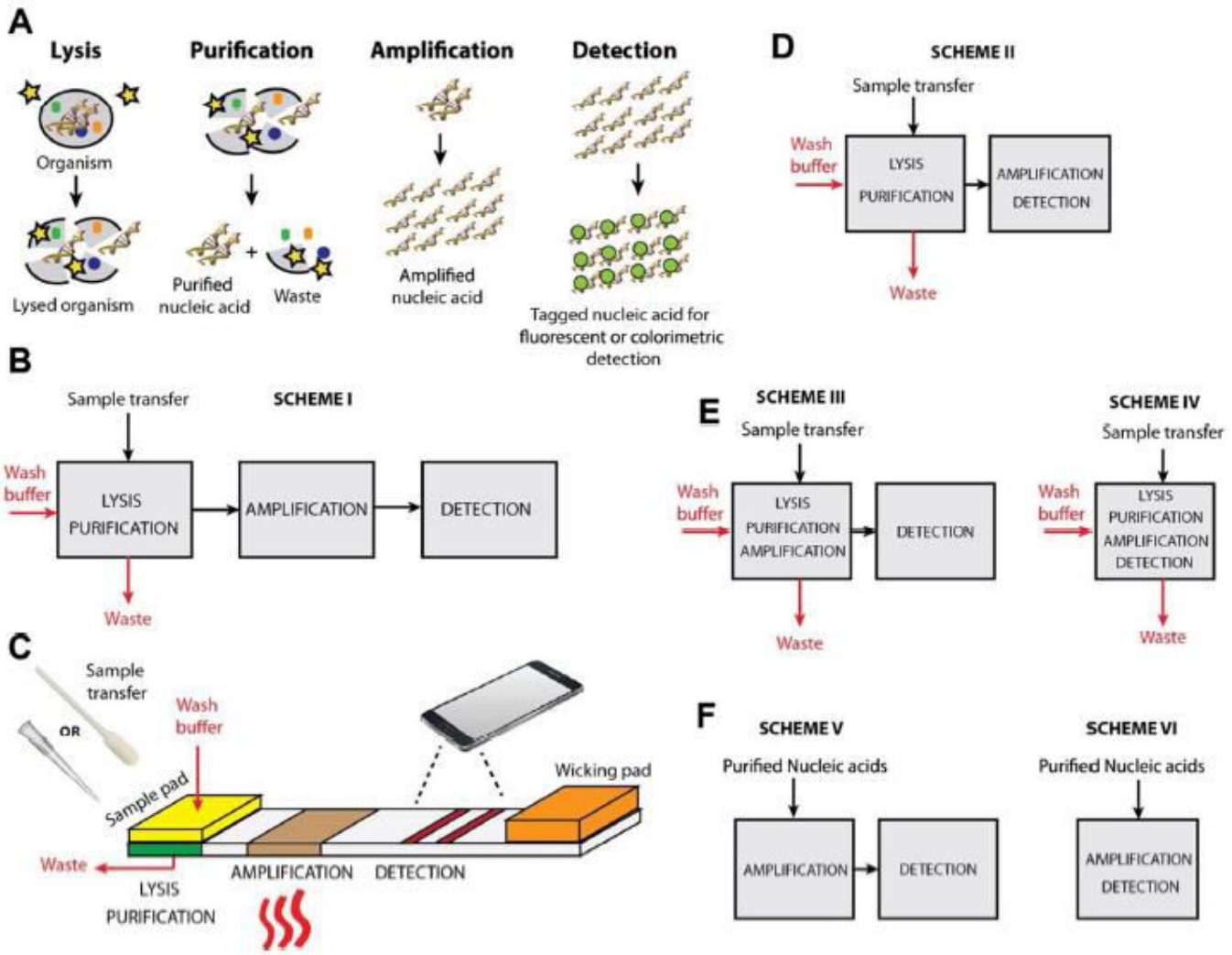
- In recent years, there has been a resurgence in the field of paper-based microfluidic device development.
- Paper can be used for:

1. **extraction,**
2. **amplification and**
3. **detection of nucleic acids.**

- The operations involved, i.e. **lysis, NA purification, and amplification**, require different reagents that must be flowed sequentially to this piece of paper, thus increasing operational complexity.
- To date, there has been **no device that has automated all these sequential operations**.
- There have been three methods of realizing sequential delivery: i) manually moving a paper reaction zone from one location to another, ii) manually adding reagents to a paper reaction zone, and iii) designing origami-based devices that may be folded in different ways to connect with the desired reagent.
- **All these methods require multiple timed user steps, which continues to be a major drawback of most current paper-based NAATs.**

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics

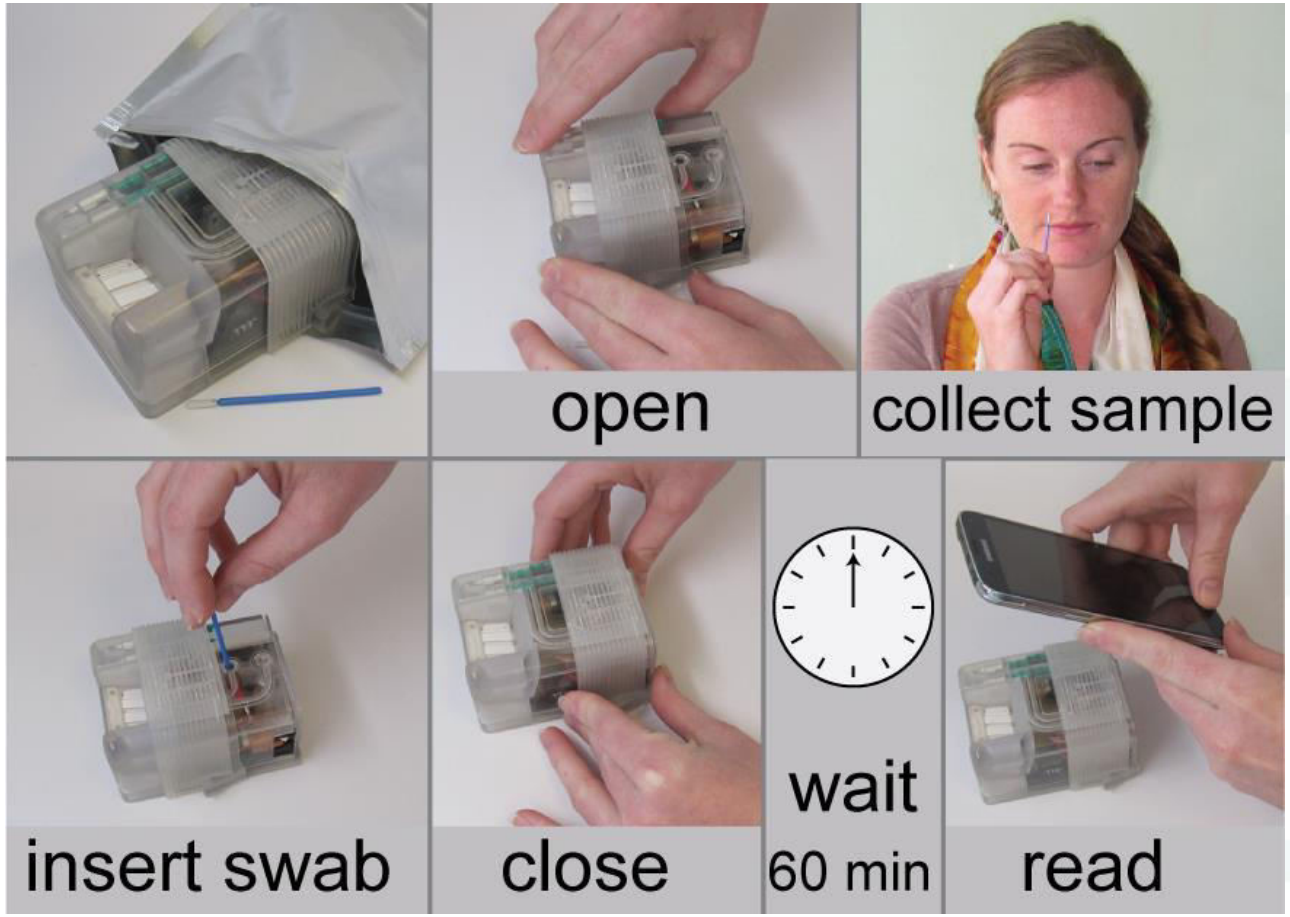


- **Goal – development of portable paper-based device.**
- Nucleic acid amplification tests (NAATs) in paper-based microfluidics.
- PCR and LAMP have been the most preferred methods for developing paper-based NAATs.
- Different options of devices – SCHEME I-VI

N. Kaur, B. J. Toley, Paper-based nucleic acid amplification tests for point-of-care diagnostics, The Analyst 143 (7), 2018

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics

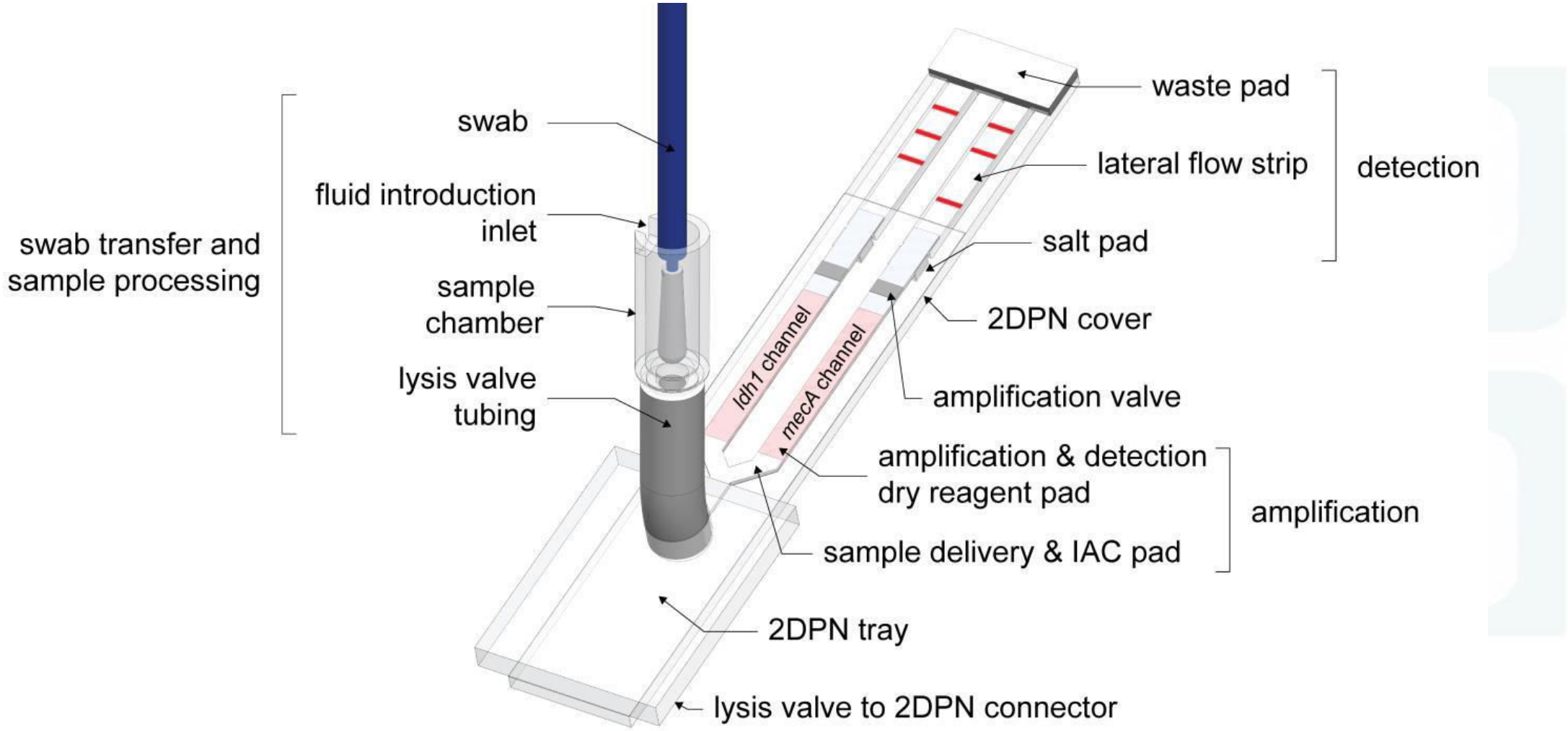


- First fully integrated NAAT in paper-based device.
- Sample collection and result within 60 minutes.
- The device was tested with patient's nasal swab sample to detect the presence of *Staphylococcus aureus.*
- All reagents were stored dry on the device and rehydration buffers were included inside the device.

L. K. Lafleur, J. D. Bishop, E. K. Heiniger, R. P. Gallagher, M. D. Wheeler, P. Kauffman, X. H. Zhang, E. C. Kline, J. R. Buser, S. Kumar, S. A. Byrnes, N. MJ Vermeulen, N. K. Scarr, Y. Belousov, W. Mahoney, B. J. Toley, P. D. Ladd, B. R. Lutz, P. Yager, A rapid, instrument-free, sample-to-result nucleic acid amplification test, Lab on a chip (2013) Vol. 1

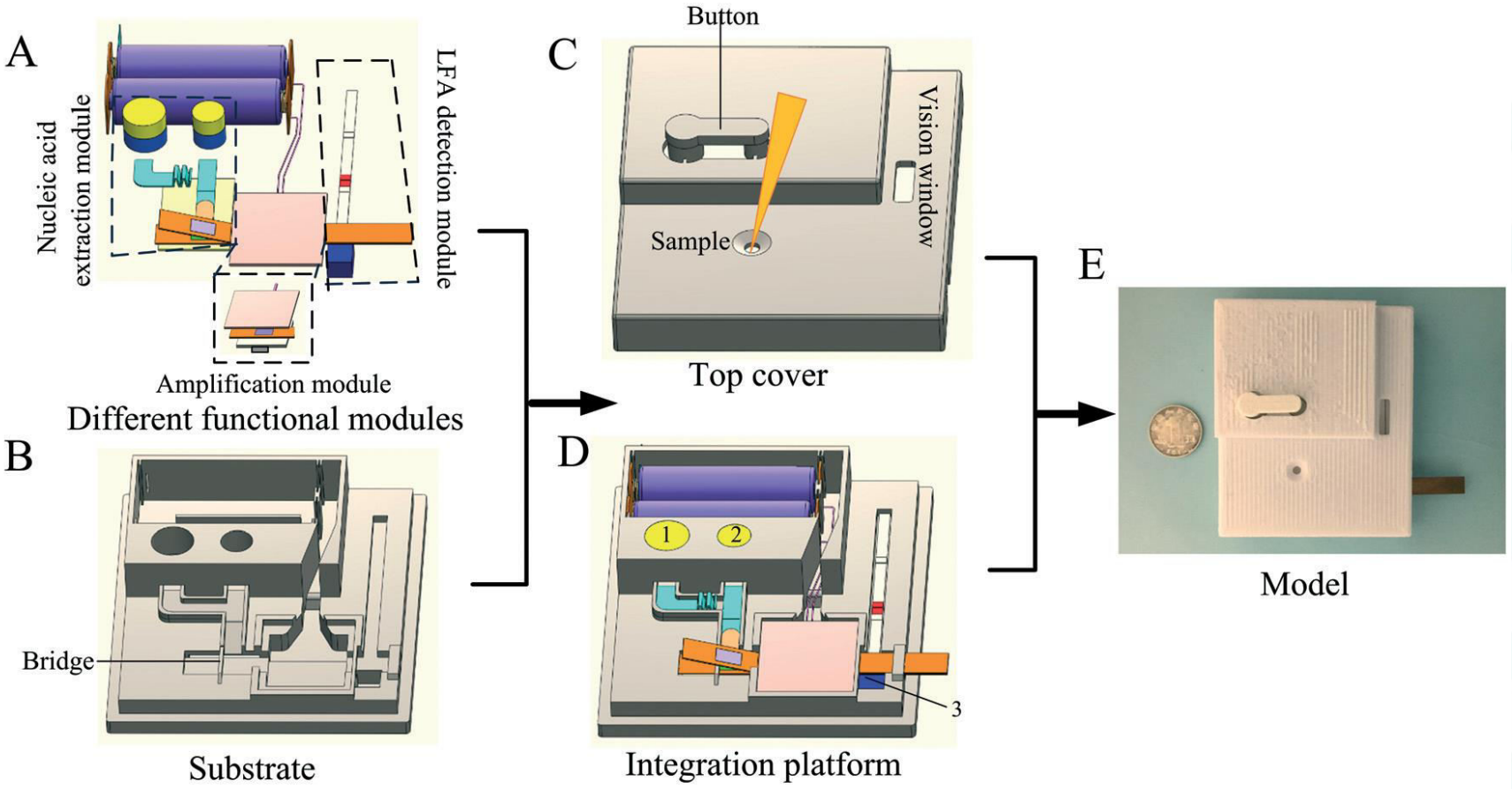
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

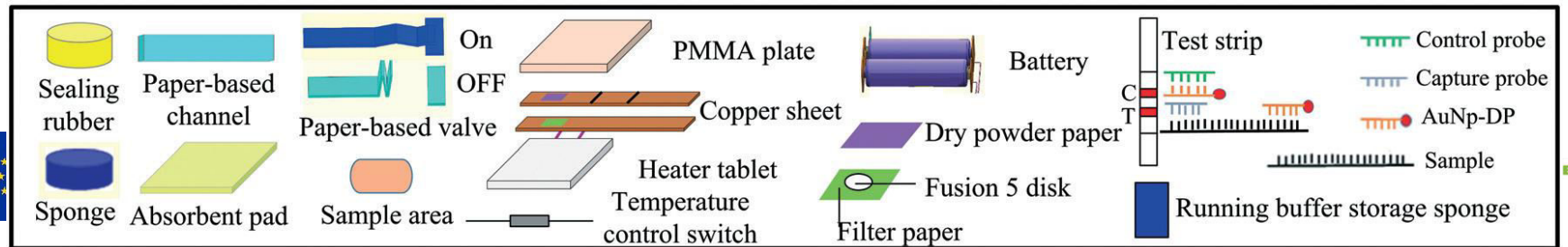
Paper-based devices molecular diagnostics



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Paper-based devices molecular diagnostics





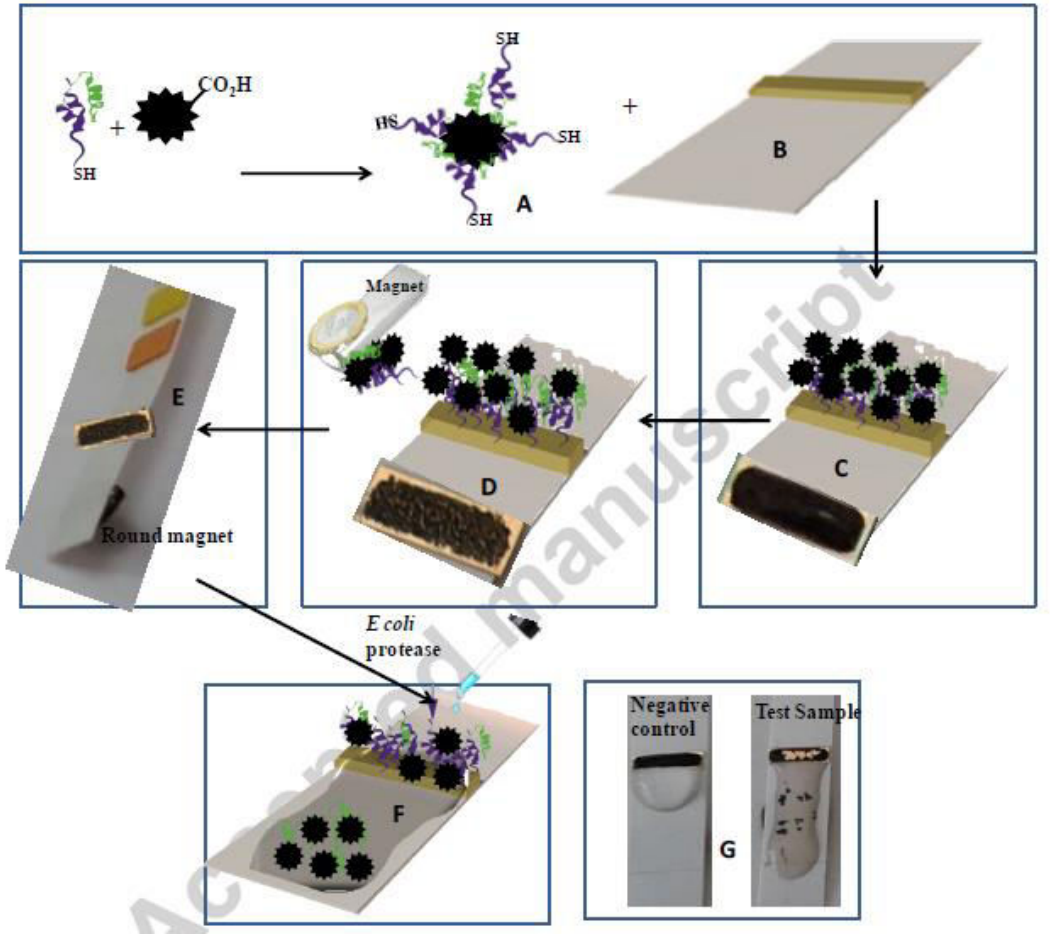
- Fully disposable and integrated paper-based device by integrating paper-based nucleic acid extraction, paper-based isothermal amplification and LFA into one paper device.

R. Tang, H. Yang, Y. Gong, M. You, Z. Liu, J. R. Choi, T. Weng, Z. Qu, Q. Mei, F. Xu, A fully disposable and integrated paper-based device for nucleic acid extraction, amplification and detection, Lab on a Chip (2017) Issue 7

Magnetic paper-based sensors for molecular diagnostics

IPANEMA

- Development of simple, sensitive, specific and cost-effective strip-format colorimetric biosensor for quantitative and qualitative detection of *E. coli* in food products.
- Hand-held peptide probe in a strip format.
- Protease peptide substrate labelled with magnetic nanoparticles (MNPs) and immobilized on a gold sensing platform.
- MNP suspension was mixed with peptide, coupling agent EDC and NHS.
- Self-adhesive tape was coated with a thin layer of gold.
- MNP-peptide solution was deposited on a gold strip and excess was removed with magnet.
- The solution of *E. coli* crude protease was down streamed over the sensing platform.

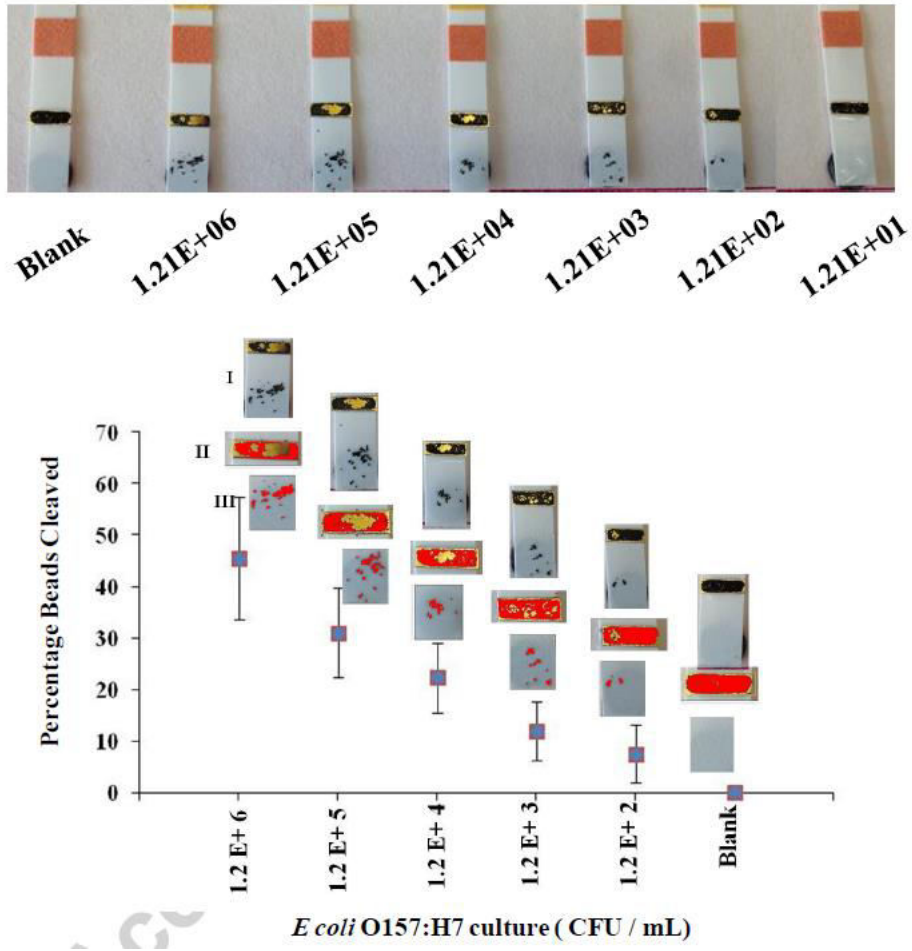


This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Magnetic paper-based sensors for molecular diagnostics

IPANEMA

- Qualitative detection – naked eye.
- Color changes from black to golden.
- Quantitative detection – ImageJ software.
- The response time was defined as the time needed for the *E. coli* protease solution to dissociate the MNP-peptide SAM, followed by attraction of these moieties to the magnet stacked at the back of the biosensor.
- The response time was around 30 seconds.
- Biosensor was successfully used for detection of *E. coli* in complex food matrices, with the limit of 30-3000 CFU/ml.
- No pretreatment or protease are needed for this process.



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Magnetic paper-based sensors for molecular diagnostics

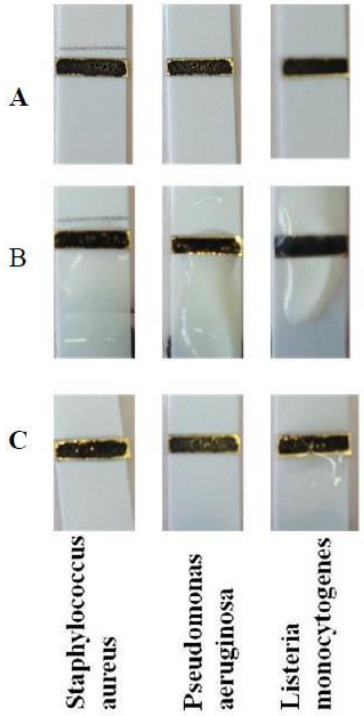


Fig. 2. Colorimetric *E. coli* protease sensor probe under the effect of other food-borne pathogens proteases. (A), Biosensor chip functionalized with MNPs–specific *E. coli* peptide substrate. (B) Functionalized biosensor incubated with different proteases. (C) Functionalized biosensor after incubation with different proteases.

- The selectivity of the probe was tested by examining the protease sensor probe under other food-borne pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*.
- *E. coli* protease sensor probe doesn't react with other food-borne pathogens.

G. A. R. Y. Suaifan, S. Alhogail, M. Zourob, Paper-based magnetic nanoparticle-peptide probe for rapid and quantitative colorimetric detection of *Escherichia coli* O157:H7, Biosensors and Bioelectronic 92 (2017)702-708.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Magnetic paper-based sensors for molecular diagnostics

IPANEMA

- The development of nanomaterial-based biosensor as a portable real time PSA diagnostic device.
- PSA – Prostate Specific antigen
- Novel, facile and rapid paper-based PSA biosensor based on the use of magnetic nanoparticles (MNPs)-peptide probe for the detection of active PSA form.
- The designed biosensor was based on the detection of PSA amidolytic activity using MNPs-peptide probe placed on top of paper support.
- Following PSA introduction, the linkage between the MNPs and the sensor gold surface will be abolished. An external magnetic field stacked at the sensing support back will collect the cleaved MNPs-peptide moieties away from the gold sensing platform with an optical color change.
- The proteolytic activity of PSA results in the dissociation of the MNPs-peptide moieties.

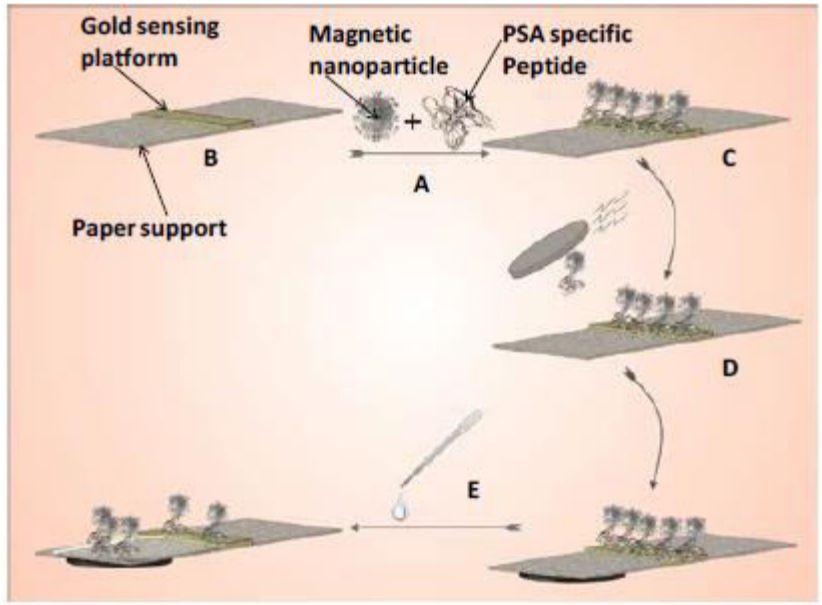
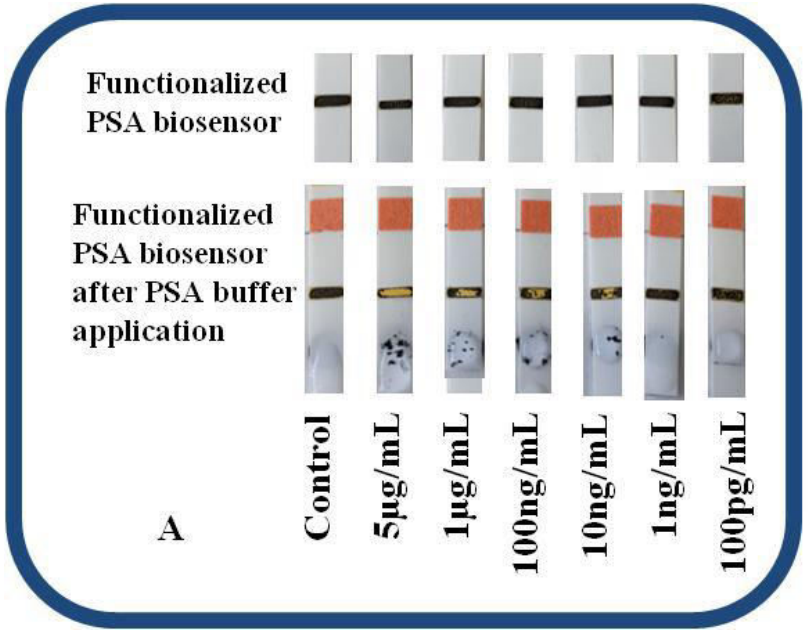


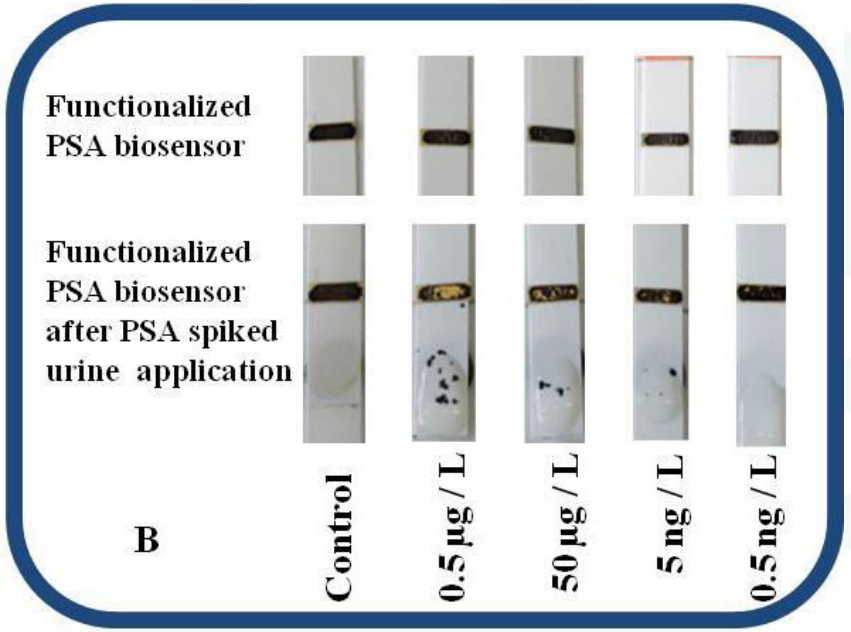
Fig.1. Mechanism of proteolytically active PSA detection using MNPs-peptide probe (**A**) Functionalisation of MNPs with PSA specific peptide substrate (**B**) Gold sensing platform. (**C**) Immobilization of functionalized MNPs on gold sensing platform placed over a paper support (**D**) Sensor platform under the effect of an external magnet to remove any unattached MNPs. (**E**). Biosensing process.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Magnetic paper-based sensors for molecular diagnostics

- Detection limit was the lowest PSA concentration incapable of cleaving peptide MNP moieties covalently attached to the gold platform.
- Detection limit for PSA buffer samples was 1 ng/ml.
- Detection limit for PSA spiked urine samples was 0.5 ng/ml





G. A. R. Y. Suaifan, M. Zourob, Paper-based biosensor for protease detection using magnetic nanoparticles, International journal for oncology and cancer therapy (2017), Vol 2.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 872662

Microfabrication Techniques for Paper Based Microfluidics

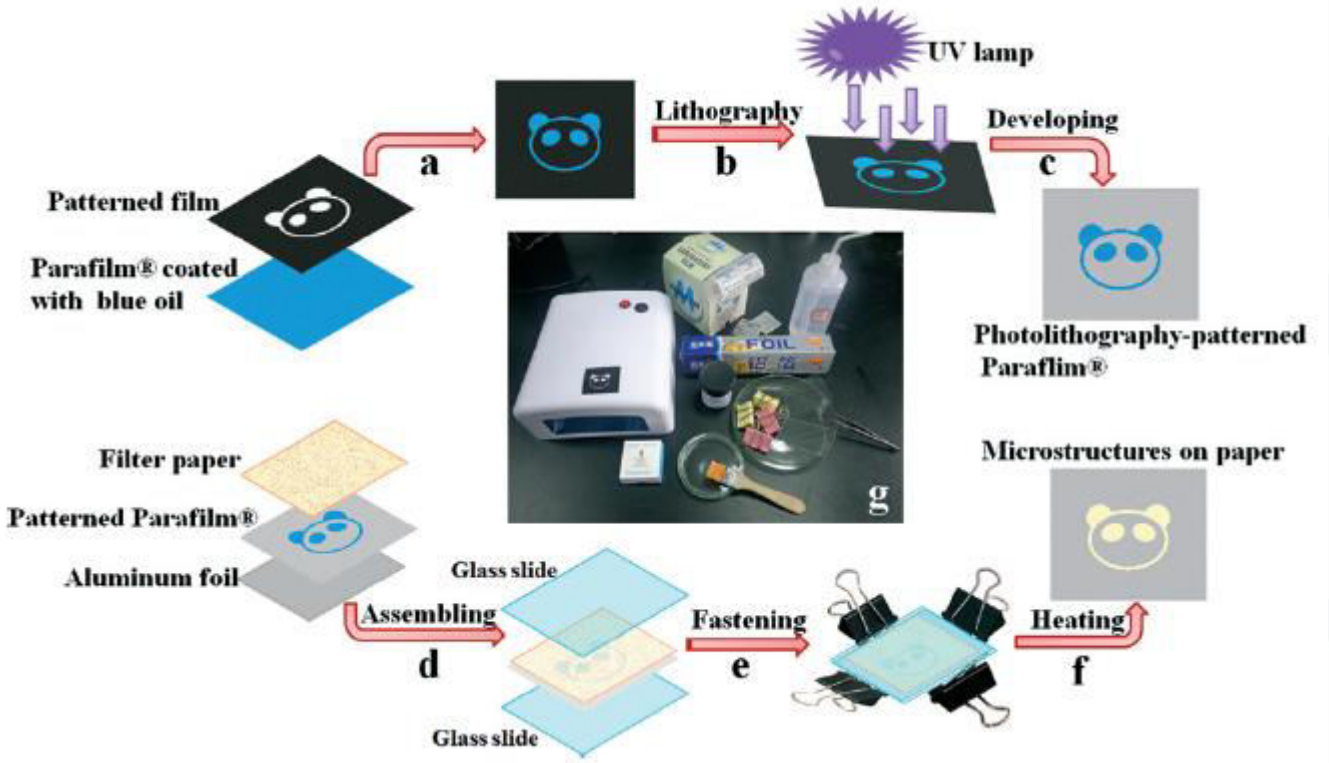
- Photolithography—areas covered with negative photoresist (e.g., SU-8) - hydrophobic, areas without photoresist – hydrophilic
- Wax printing - the heated wax melts and spreads vertically and laterally into the paper
- PDMS dispensing—PDMS barriers are printed on filter paper after dissolved with hexanes
- Plasma treatment—the plasma treated areas are strongly hydrophilic

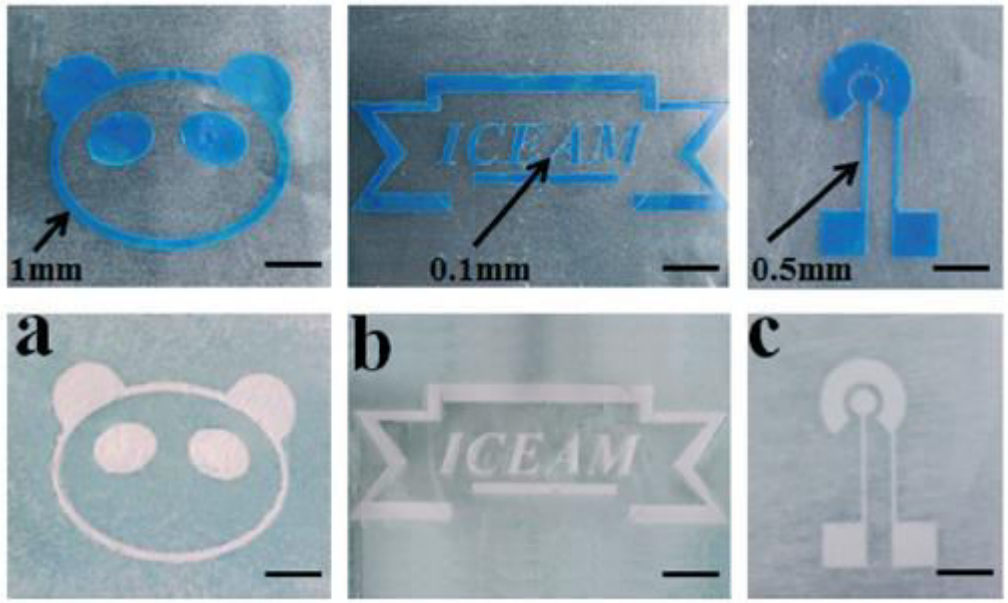
F.J. Galindo-Rosales (ed.), *Complex Fluid-Flows in Microfluidics*,
Vania Silverio and Susana Cardoso de Freitas,
Microfabrication Techniques for Microfluidic Devices

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Photolithography and embossing of Parafilm

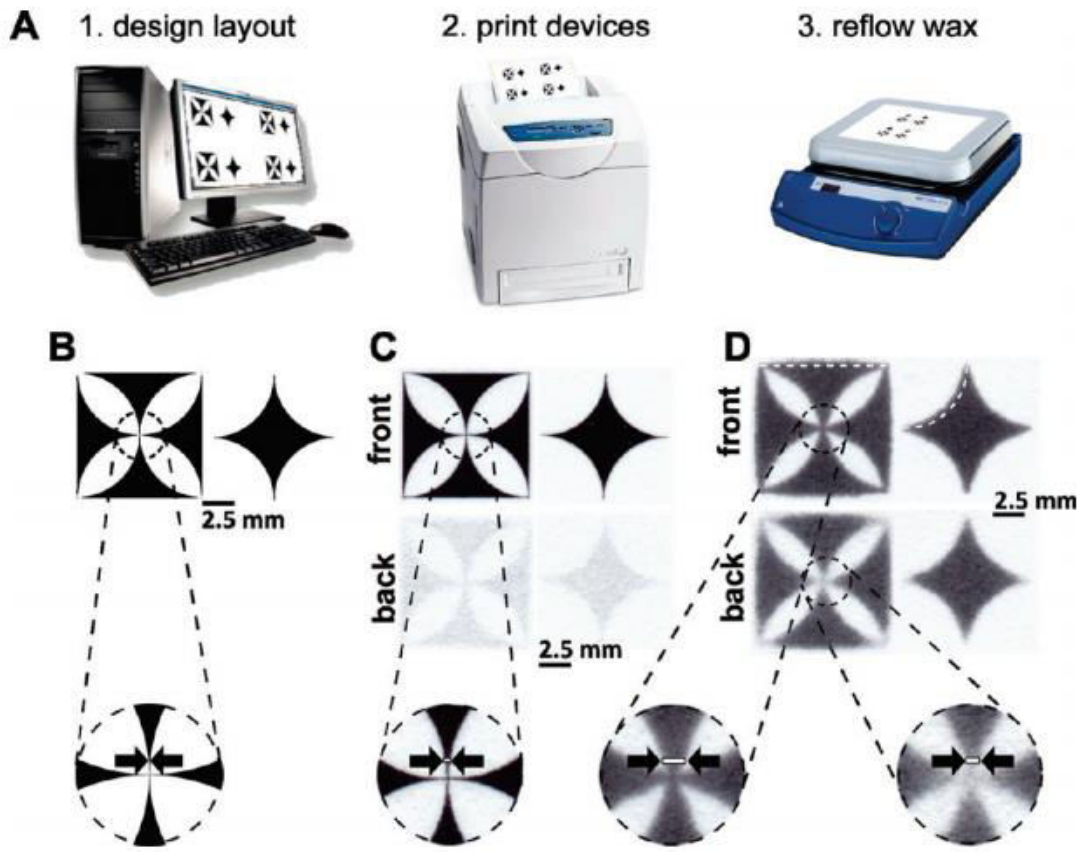
- Ling Yu and Zhuan Zhuan Shi, Microfluidic paper-based analytical devices fabricated by low-cost photolithography and embossing of Parafilm, Lab Chip, 2015, 15, 1642



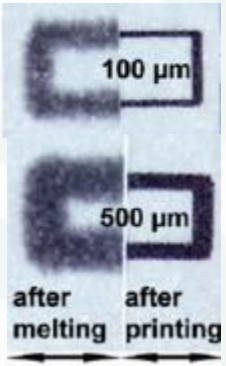


Wax Printing

- Emanuel Carrilho, Andres W. Martinez, and George M. Whitesides, Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics, Anal. Chem. 2009, 81, 7091–7095



Molten Wax spreading model



$$L = (\gamma D t / 4\eta)^{1/2}$$

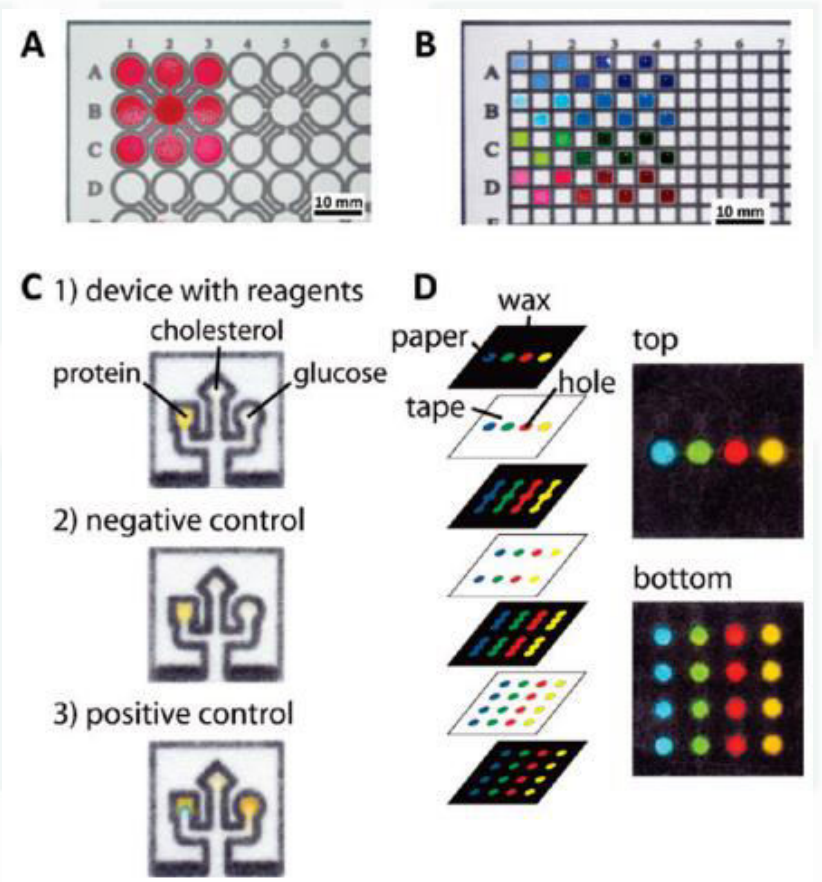
where L is the distance that a liquid of viscosity η and surface tension γ penetrates a porous material with an average pore diameter D in time t

Whatman chromatography paper

µ-PADs

Emanuel Carrilho, Andres W. Martinez, and George M. Whitesides, Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics, Anal. Chem. 2009, 81, 7091–7095

- Paper-based multizone plates (A, B), lateral-flow devices (C), and three-dimensional µ-PADs (D);
- (A) A 96-zone plate with microfluidic channels for sample distribution.
- (B) A 384-zone paper plate after application of 1-8 µL of several dyes.
- (C) A µPAD for detecting total protein, cholesterol, and glucose in biological fluids. The reagents for each assay were added to each test zone before the device was used. The negative control wicked a phosphate buffer saline solution (PBS), while the positive control wicked a solution containing 15 µM bovine serum albumin (BSA), 40 mM cholesterol, and 5 mM glucose in PBS.
- (D) 3D µPADs by stacking layers of patterned paper and double-sided adhesive tape. The device distributes four individual samples (we show aqueous dyes) from inlets on the top of the device into an array of 16 test zones on the bottom of the device



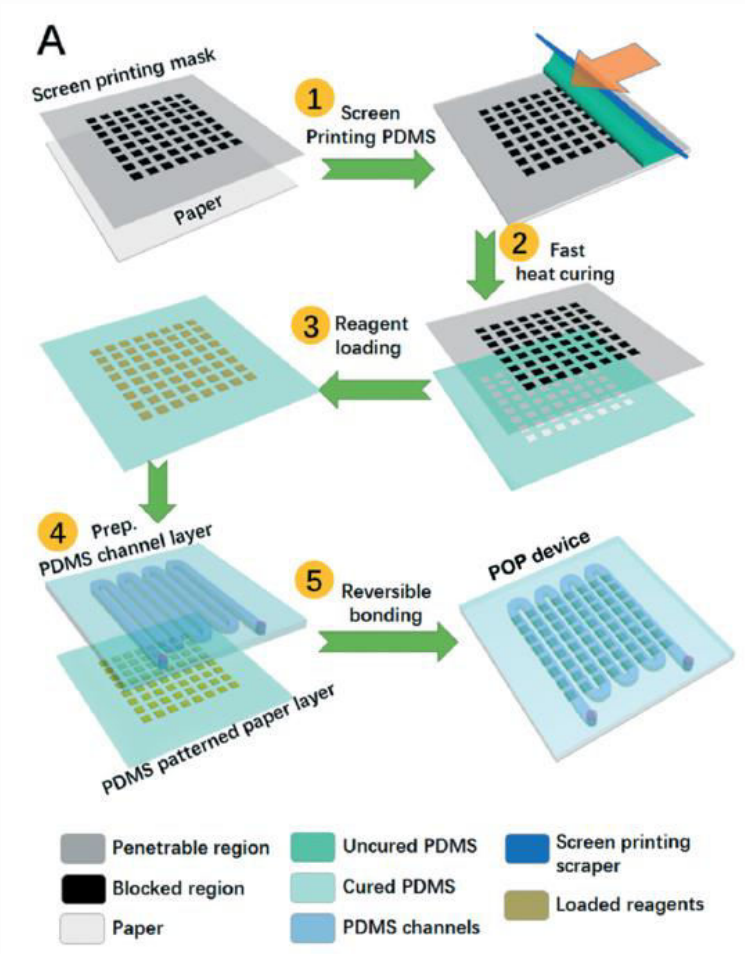
PDMS on paper (POP)

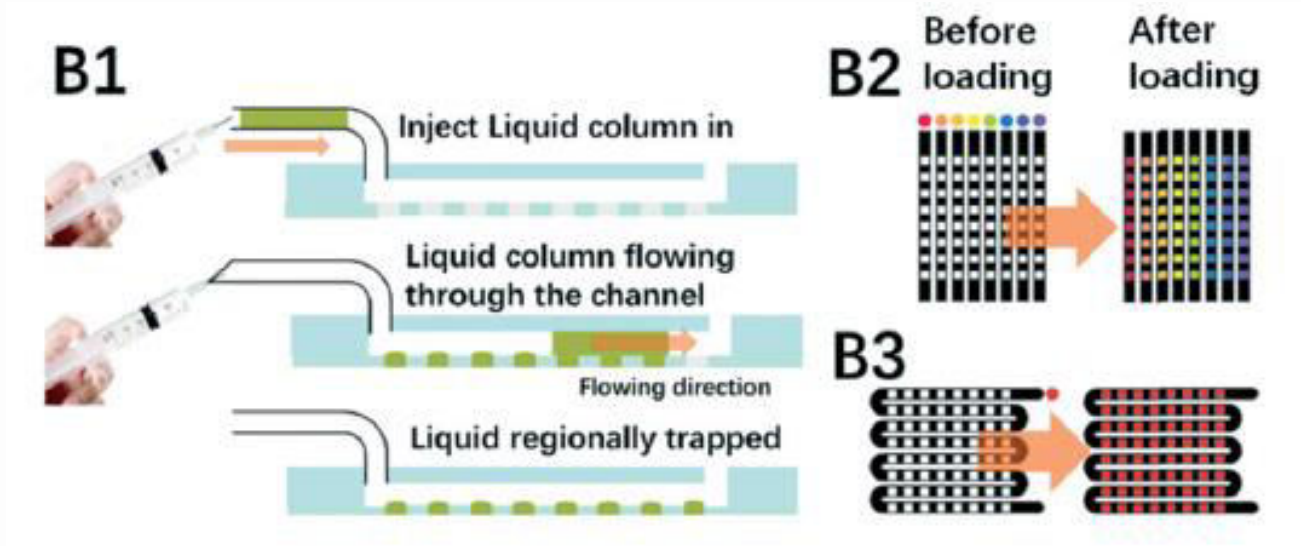
- Jin-Wen Shangguan, Yu Liu, Jian-Bin Pan, Bi-Yi Xu, Jing-Juan Xu and Hong-Yuan Chen, Microfluidic PDMS on paper (POP) devices, Lab Chip, 2017, 17, 120
- PDMS:
  - highly biocompatible, transparent, stretchable
  - difficulties in surface modification
- Paper:
  - easy surface modification
  - hydrophilic and rich with fibers
  - easy to immobilize reagents
  - capillary flow

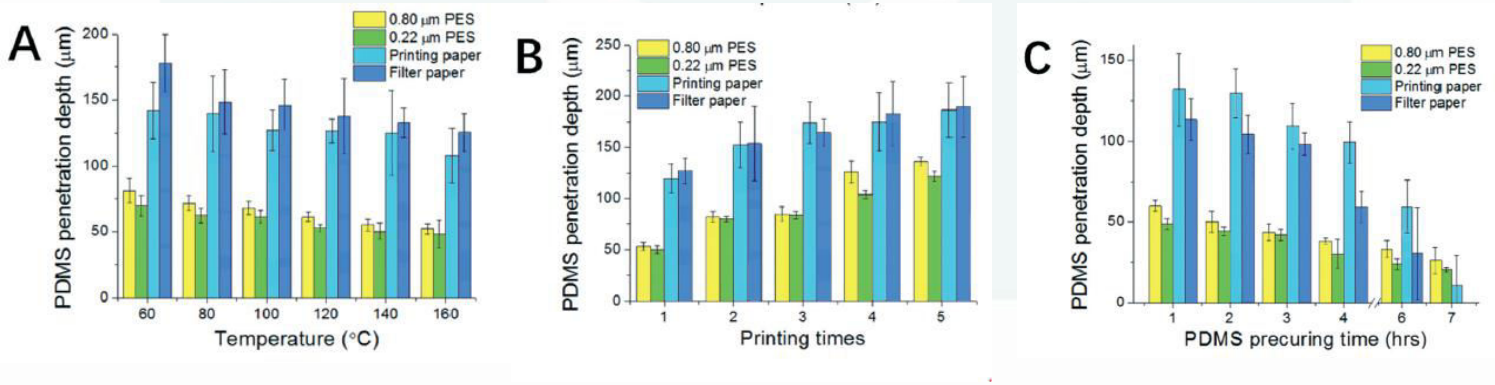
Combination of **channel based** microfluidics and **paper based** microfluidics

- The types of paper PES-80 (0.80 µm pore size) and PES-22 (0.22 µm pore size) paper, printing paper and filter paper

PDMS on paper (POP)





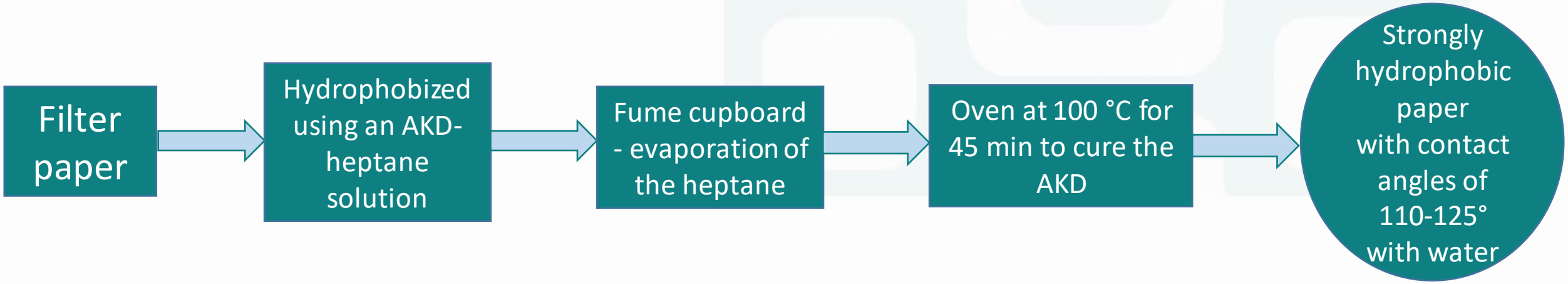


Jin-Wen Shangguan, Yu Liu, Jian-Bin Pan, Bi-Yi Xu, Jing-Juan Xu and Hong-Yuan Chen, Microfluidic PDMS on paper (POP) devices, Lab Chip, 2017, 17, 120

Plasma treatment

Xu Li, Junfei Tian, Thanh Nguyen, and Wei Shen, Paper-Based Microfluidic Devices by Plasma Treatment, *Anal. Chem.* 2008, *80,* 9131–9134

- Drawbacks of the previous technologies:
  - Photoresist is less flexible than paper, and the device can be easily damaged by bending and folding;
  - Paper is not a uniform porous material - the wall of the barrier pattern is not straight.
- Paper substrate - Whatman filter paper

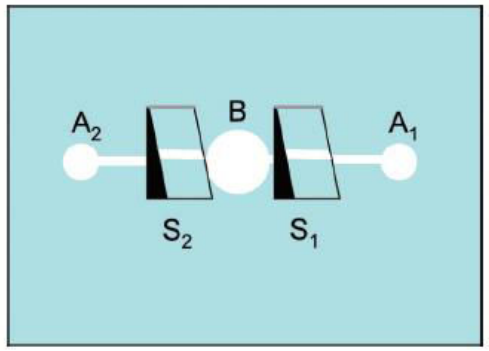


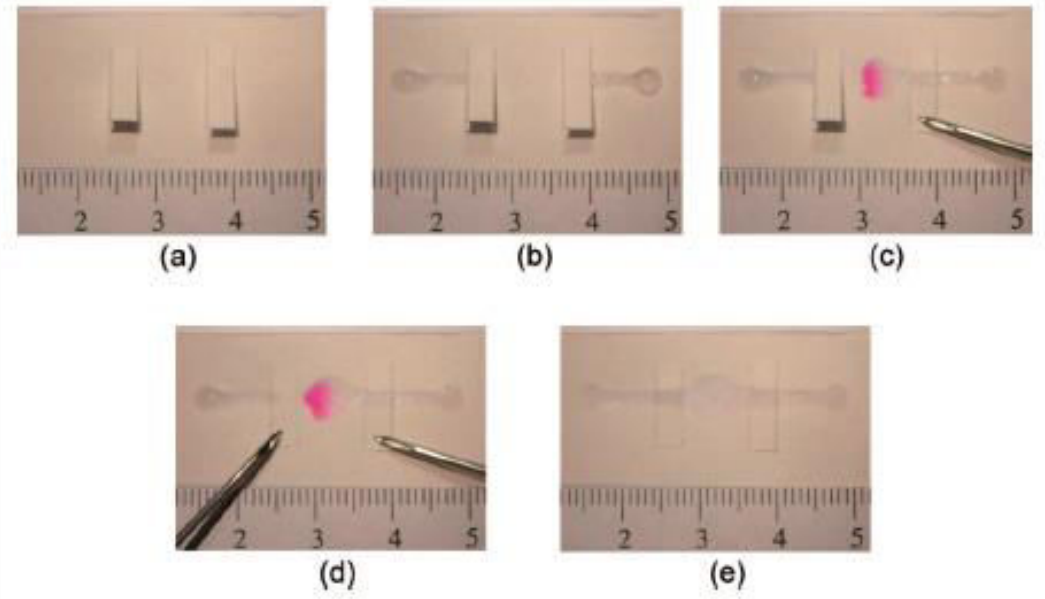
AKD - Alkyl ketene dimer

Plasma treatment

Xu Li, Junfei Tian, Thanh Nguyen, and Wei Shen, Paper-Based Microfluidic Devices by Plasma Treatment, *Anal. Chem.* 2008, *80,* 9131–9134

- Hydrophilic patterns formed on paper samples using plasma treatment;
- The plasma treatment does not leave visible mark on the samples, which retained its original flexibility;
- The plasma treated areas - strongly wettable by water or aqueous solutions;

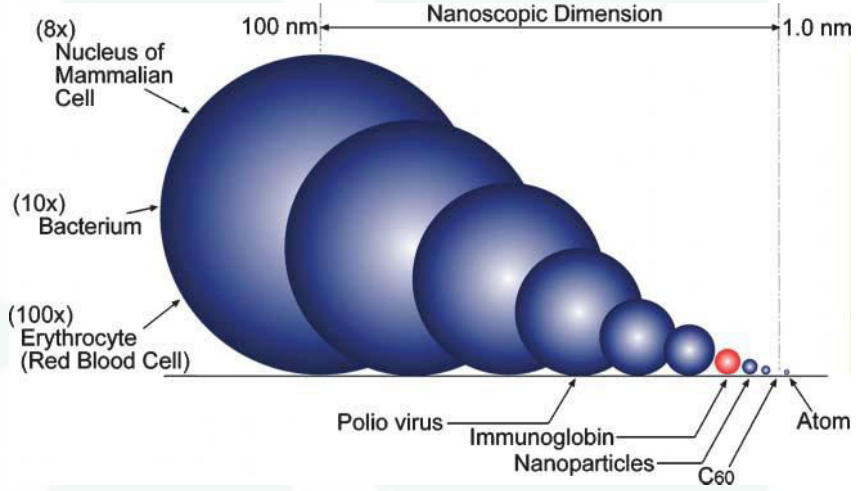




(a) Phenolphthalein was deposited in the reaction site B. (b) Dosing of a NaOH solution via A1 and HCl solution via A2; (c) NaOH solution was allowed into the reaction site, triggering indicator color change; (d) HCl solution was allowed into the reaction site, causing indicator color fading; (e) complete reversion of indicator color by HCl solution.

Nano(bio)technology

- Detection of bacteria is based upon fundamental characteristics like morphological and/or molecular chemistry of cells.
- Innovative, rapid, sophisticated, high sensitive detection methods are demanded.
- Conventional and current molecular diagnostics (ELISA, PCR, micro array) – high sensitivity and reliability, but high cost of performance, sample pretreatment, lower limit of detection.
- The drawbacks can be overcome by nanoscience.
- **Nanobiotechnology** – nanomaterials are fabricated and used for biological and biochemical applications.
- Use of nanobiotechnology extends the limits of current molecular diagnostics, allows point-of-care diagnostics, enables diagnosis at single-cell and molecule level.



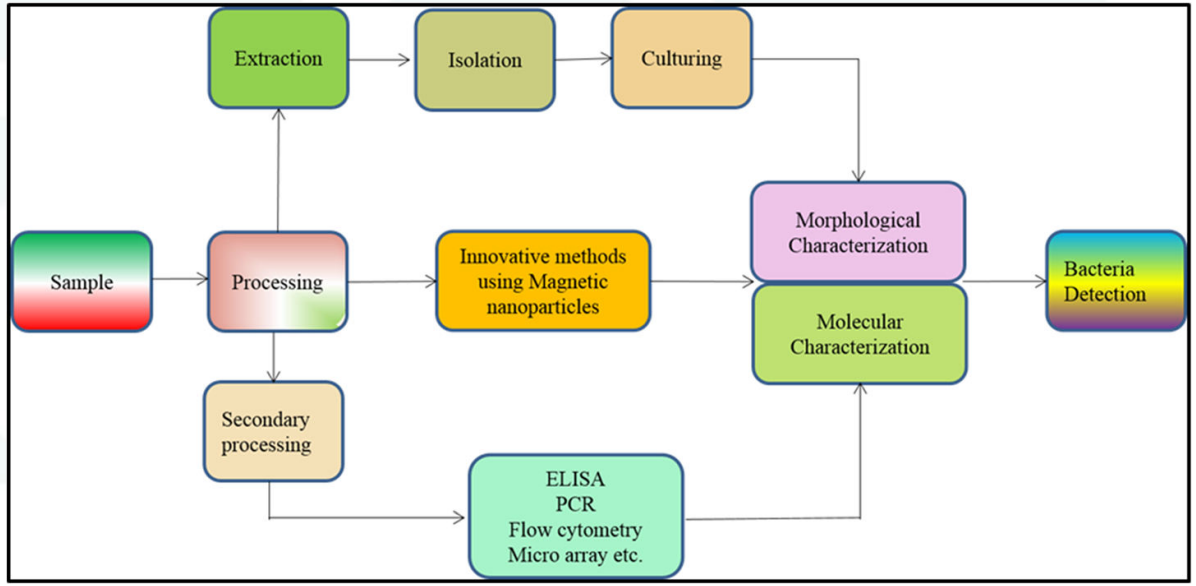
Hongwei Gu, Keming Xu, Chenjie Xu and Bing Xu, Biofunctional magnetic nanoparticles for protein separation and pathogen detection, *Chem. Commun*. (2006), 941-949

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Magnetic nanoparticles (MNPs)

- The unique physiochemical properties of MNPs such as size, magnetization level and surface morphology are complementary for biological applications.
- Rapid and real-time detection with small sample volumes is possible due to large surface/volume ratio.
- Size range from nanometers to tens of nanometers made them get closer to a biological entity of interest.

- Functionalization enables them to bind or interact with a biological target.
- The large number of target-specific molecules of interest can be attached to a large surface area provided by nanoparticles for ultra-sensitive detection.



Raghvendra A. Bohara and Shivaji H. Pawar, Innovative Developments in Bacterial Detection with Magnetic Nanoparticles, *App Biochem Biotechnol* 176 (2015) 1044-1058

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

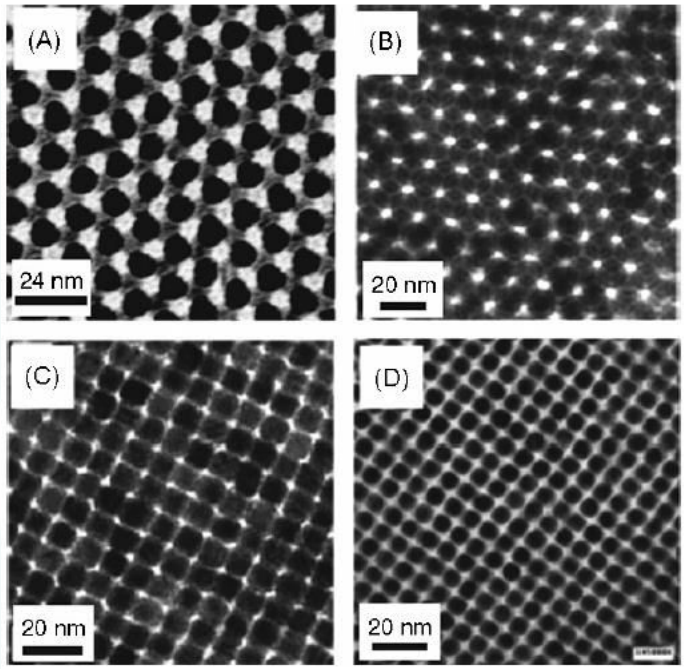
Physicochemical properties of MNPs

1. Composition and size

- Mainly consist of magnetic elements such as iron, nickel and cobalt, and their chemical compounds.
- Size (5-500 nm) – influencing the physical stability. The response of MNPs under magnetic fields changes along with the change of the size.

2. Surface properties

- As the particle size decreases, the special surface area increases (surface and interface properties become more and more significant).
- Surface spins greatly influence magnetization.



Assemblies of monodisperse nanoparticles of :
A) 8 nm Co, B) 13 nm Fe, C) 10 nm Fe_3O_4 and D) 6 nm FePt

Chenjie Xu and Shouheng Sun, Monodisperse magnetic nanoparticles for biomedical applications-Mini Review, *Polymer International* 58 (2007) 821-826

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Physicochemical properties of MNPs

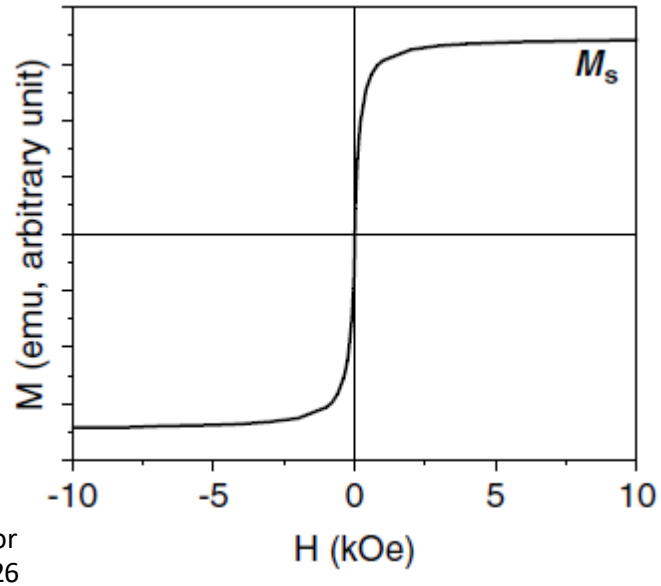
3. Magnetic properties

- Magnetic susceptibility (χ) – magnetization (M)/magnetic field (H); χ=μ-1
- Magnetic permeability (μ) – magnetic induction (B)/ magnetic filed (H)
- Ferromagnetism (χ>>0) – NPs show magnetism even if they are not in a magnetic field, and after magnetization they show permanent magnetism; paramagnetism (χ>0) – NPs show magnetism in a magnetic field and no magnetism remains when the magnetic field is removed; diamagnetism (χ<0) – NPs do not show magnetism whether or not they are in a magnetic field

superparamagnetic NPs < critical size of MNPs > ferromagnetic NPs

Superparamagnetic NPs – combine the advantages of the paramagnetic and the ferromagnetic NPs, have the excellent magnetism and excellent colloidal stability.

- Hysteresis loop (magnetic moment, magnetic remanence) and the magnetization curve (particle size, monodispersity).



Chenjie Xu and Shouheng Sun, Monodisperse magnetic nanoparticles for biomedical applications-Mini Review, *Polymer International* 58 (2007) 821-826

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Designing of MNPs

Synthesis methods and conditions define morphology and structure of MNPs, that means also the properties of MNPs.
While synthesizing MNPs, attention should be given to monodispersity and reproducibility of NPs.

Synthesis

- Chemical synthesis – co-precipitation, sol-gel, thermal decomposition, hydrothermal synthesis, micro-emulsion, combustion and polyol synthesis.
- Biological synthesis – clean, non-toxic and eco-friendly method

Surface Modifications – protection of MNPs, improved stability, increased water dispersibility, provided functionalization for further conjugation with bioactive molecules or targeting ligands.

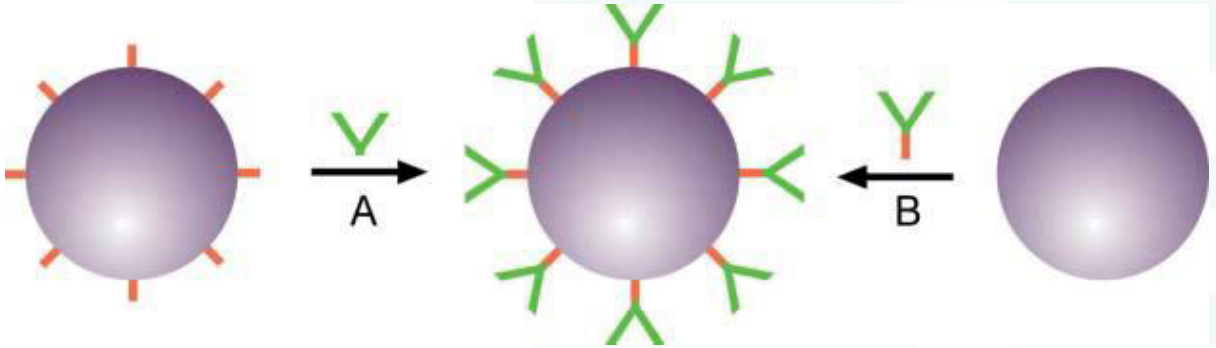
- Surface coating – polymeric stabilizers, atomic layers of inorganic metals, nonmetals or oxide surfaces
- Polymeric shells – encapsulation of MNPs in polymeric shell

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Anchoring biofunctional molecules onto MNPs

- A – a monolayer of molecules that bear a reactive group grows on the nanoparticles first; then, the biofunctional molecules react with the monolayer to yield the biofunctional nanoparticles. Simple and versatile way, but may leave unconsumed reactive groups.

- B – the group that reacts with the surface is conjugated with the biofunctional molecule first; then, the conjugate reacts with the nanoparticle to give the desired product. Produces a well-defined monolayer, but it sometimes requires considerable effort to engineer a biofunctional molecule that bears a surface reactive group.

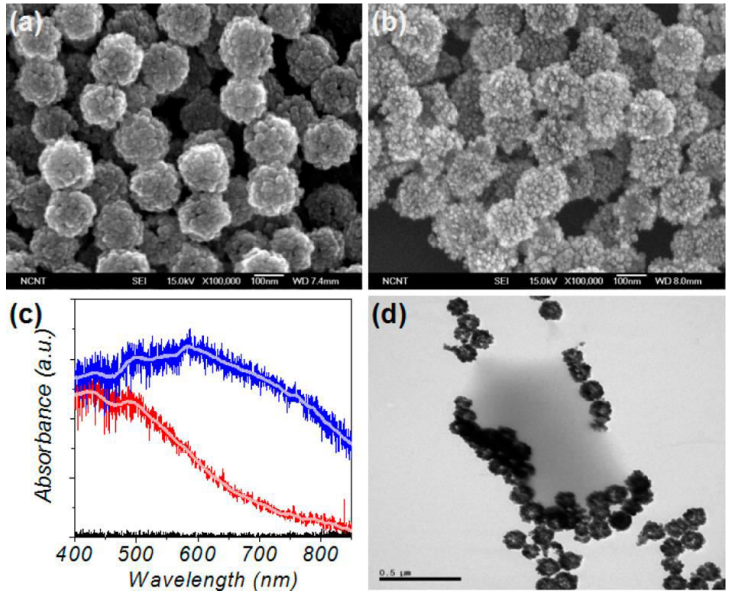


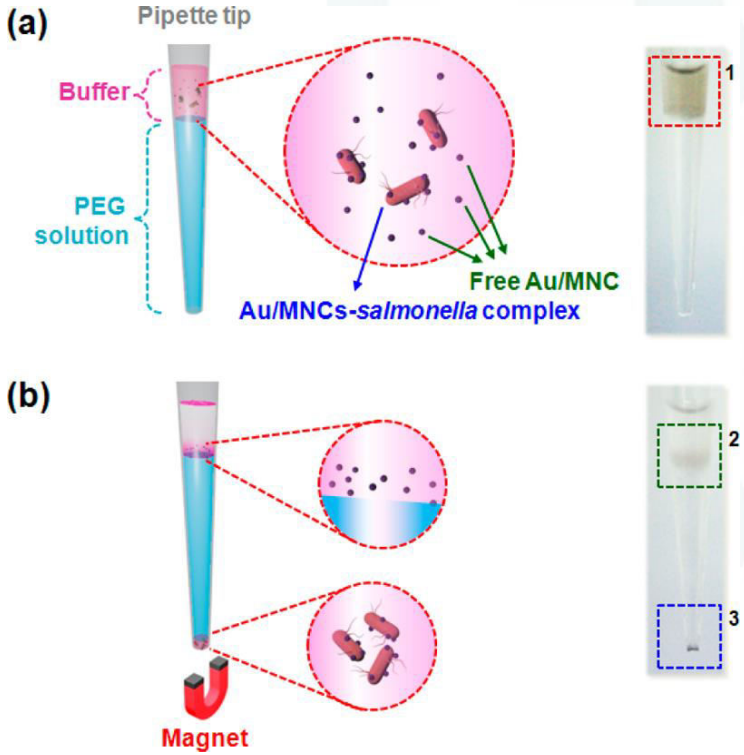
Hongwei Gu, Keming Xu, Chenjie Xu and Bing Xu, Biofunctional magnetic nanoparticles for protein separation and pathogen detection, *Chem. Commun.* (2006), 941-949

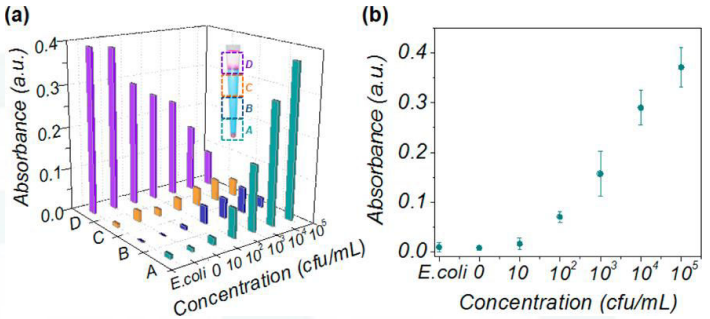
This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

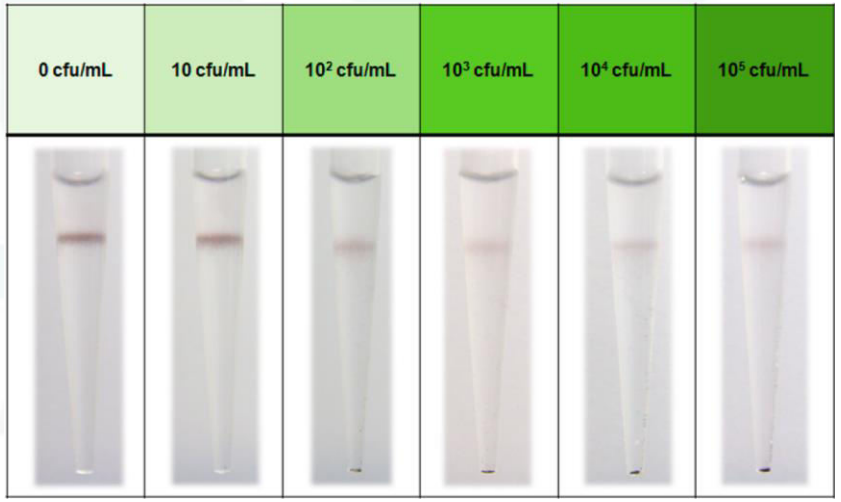
Pathogen detection using MNPs

D. Kwon, J. Joo, J. Lee, KH. Park, S. Jeon, Magnetophoretic Chromatography for the Detection of Pathogenic Bacteria with the Naked Eye, *Anal. Chem.* 85 (2013), 7594-7598





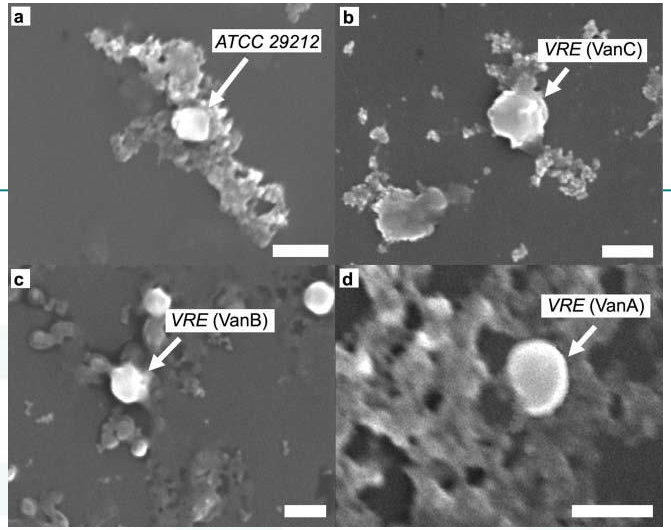


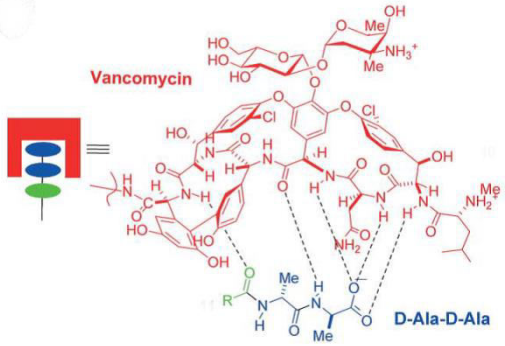


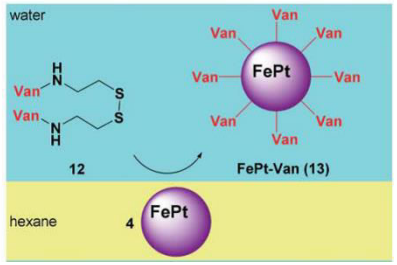
This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

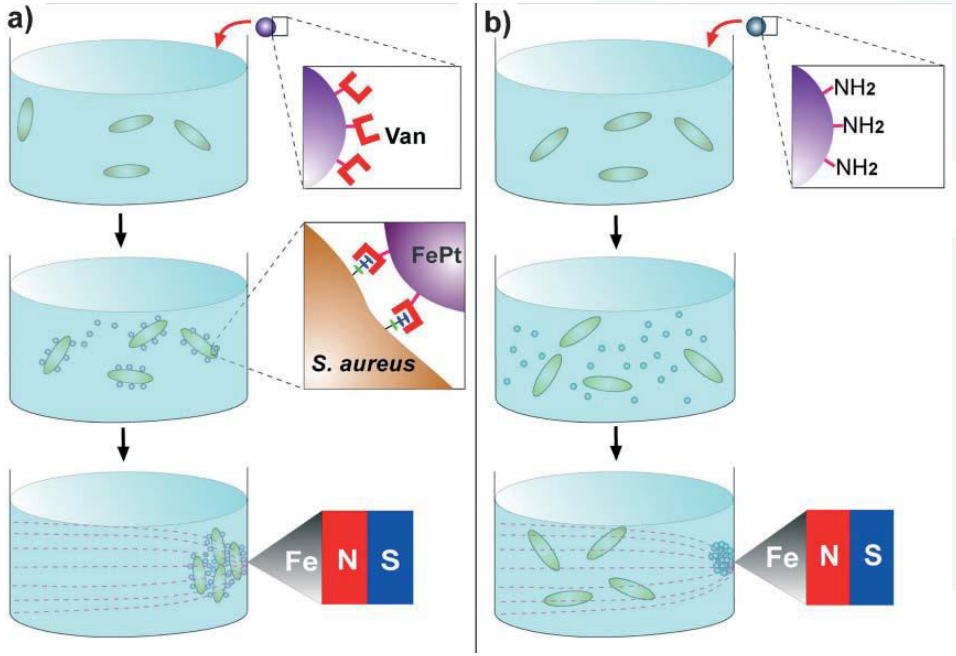
Pathogen detection using MNPs

Hongwei Gu, Keming Xu, Chenjie Xu and Bing Xu, Biofunctional magnetic nanoparticles for protein separation and pathogen detection, *Chem. Commun*. (2006), 941-949









Exp. no.	Bacteria	Type	Read-out method	Concentration/ cfu mL^{-1}
1	*S. aureus*	G(+)	SEM	8
2	*S. epidermidis*	G(+)	SEM	10
3	*S. epidermidis*	G(+)	SEM	15[a]
4	Coagulase negative staphylococci	G(+)	SEM	4
5	Coagulase negative staphylococci	G(+)	FL[b]	6
6	*E. faecalis* (ATCC 29212)	G(+)	SEM	26[c]
7	*E. gall* (VanC)	G(+)	SEM	84[c]
8	*E. faecium* (VanB)	G(+)	SEM	22[c]
9	*E. faecium* (VanA)	G(+)	SEM	34[c]
10	*Streptococcus pneumoniae*	G(+)	FL[b]	4
11	*E. coli*	G(−)	SEM	15
12	*E. coli*	G(−)	FL[b]	10

[a] Mixed with white blood cells whose concentration is 100 cfu mL^{-1}. [b] FL = fluorescence microscope. [c] Concentrations lower than indicated have not been tested.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Toxic cyanobacteria detection

- Cyanobacteria are a natural part of phytoplankton assemblages in lakes and reservoirs and commonly are present in at least low abundances.
- Multiple toxin types can be found at a single location / Several strains can produce the same toxin type
- Multiplexing may provide an answer to these issues.
- The greatest risk of exposure to elevated concentrations of cyanobacterial toxins is through accidental ingestion and inhalation during recreational activities.

World Health Organization (WHO) Guidelines for Safe Recreational Water Environments. WHO; Geneva, Switzerland: 2003. Volume 1: Coastal and Fresh Waters:

1) Low probability of adverse health effects ≤ 20,000 cells/mL
2) Moderate probability of adverse health effects 100,000 cells/mL
3) High probability of adverse health effects from contact with, ingestion, or inhalation of cyanobacteria when algal scum appears on the water surface

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

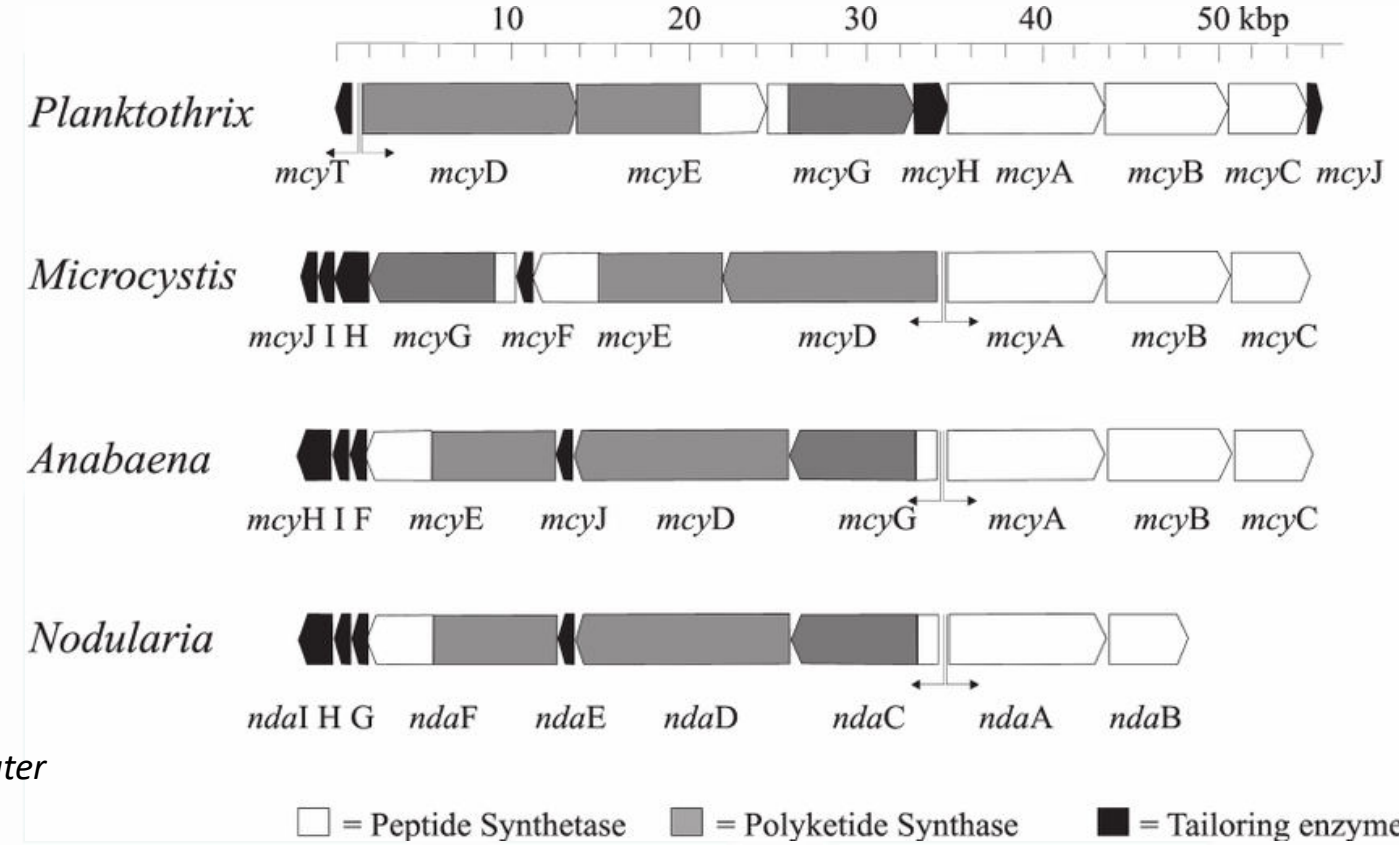
Microcystins

- The discovery of toxin biosynthetic genes opened up new possibilities for method development.

- Amongst the hepatotoxin-producing cyanobacteria, microcystin producers have been studied in the greatest detail using real-time PCR.

✓ Microcystin-producing cyanobacteria are widely distributed and cause water quality incidents in many countries.

✓ Molecular genetics of microcystin production is well understood.



World Health Organization (WHO) *guidelines for drinking-water quality*, 2nd ed. Geneva.2003.:

Low risk > 10 (µg/L)
Moderate risk 10–20 µg/L
High risk 20–2000 µg/L
Very high risk < 2000 µg/L

Target gene	Cyanobacterial genus	Main findings	Reference
mcyA	*Microcystis*	Discrimination of toxic and non-toxic Microcystis strains achieved	Foulds *et al.*, 2002
mcyE	*Anabaena, Microcystis*	Established a positive correlation between *mcyE* copy numbers and MC conc.	Vaitomaa *et al.*, 2003
mcyE	*Anabaena, Microcystis*	Development of a Chip Assay and Quantitative PCR for Detecting Microcystin Synthetase E Gene Expression	Sipari *et al.*, 2010
mcyE	*Microcystis, Anabaena,Planktothrix*	Multiplex detection with genus-specific primers and probes. *mcyE* conc. Correlated positively with environmental MC conc.	Ngewa *et al.*, 2014a
mcyB	*Microcystis*	Positive correlation between MC cell counts, , copy numbers and MC conc.	Kurmayer and Kutzenberger,2003
mcyB, mcyE	*Microcystis*	Positive correlation between *mcyB* and *mcyE* copy numbers and environmental conc. of MC	Conradie and Barnard, 2012
mcyE or *ndaF*	*Anabaena, Microcystis, Planktothrix, Nostoc, and Nodularia*	Effective discrimination between the tested cyanobacterial genera, relatively high detection rate	Rantala *et al.*, 2008

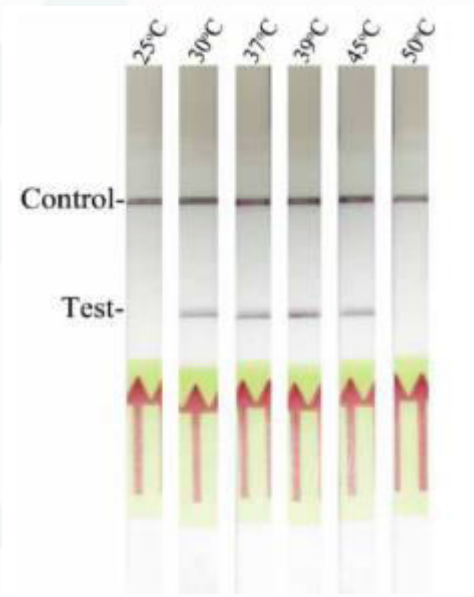
Jingjing Li, Chunming Wang, Xin Yu, Huirong Lin, Chen Hui, Li Shuai, Shenghua Zhang; Rapid detection of Cyanobacteria by recombinase polymerase amplification combined with lateral flow strips. *Water Supply* 1 June 2019; 19 (4): 1181–1186.

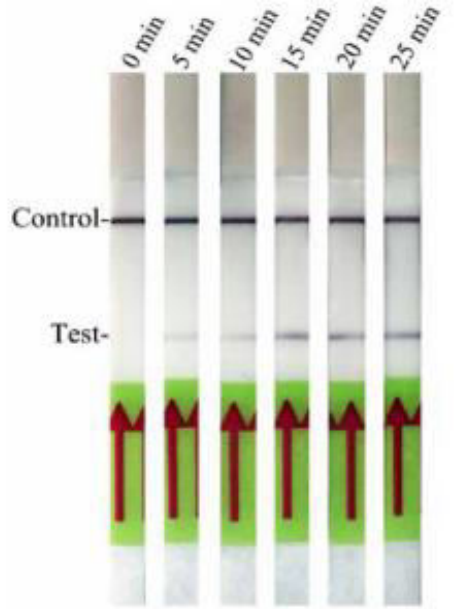
Sample preparation and nucleic acid amplification

Identifying the optimal reaction temperature

Identifying the optimal incubation duration

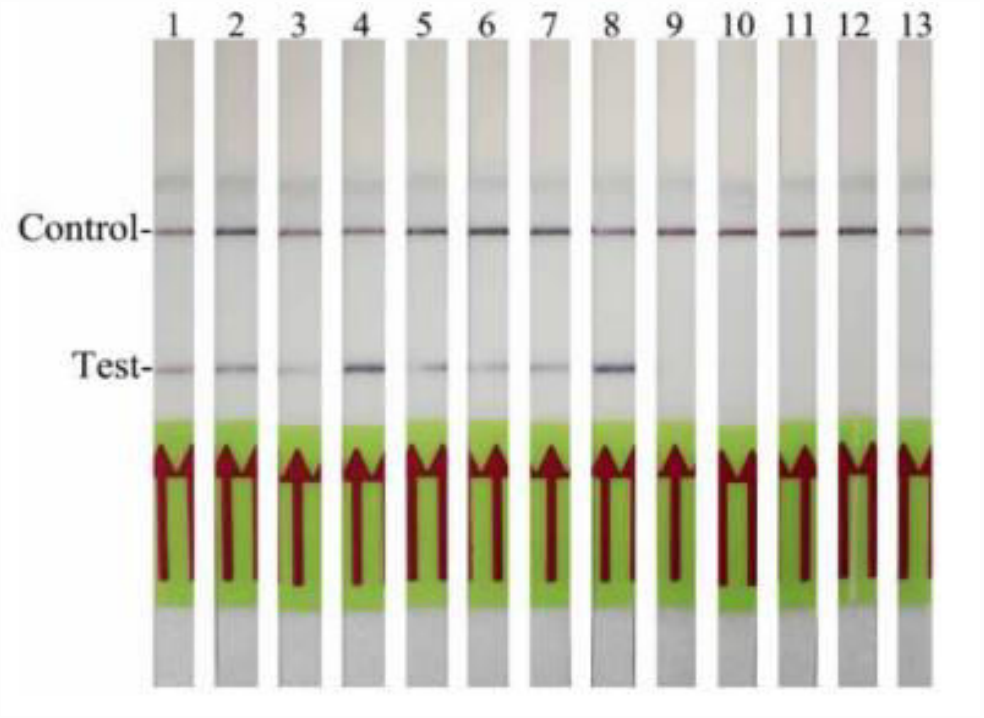
- **Cell disruption / DNA extraction –** freeze-cracking method
- **Isothermal amplification** - RPA (37°C, 15-25min)
- A 10 µl sample of this solution was transferred to the **LF strip** and incubated in a buffer at room temp.
- The color test band indicated that amplification of DNA was successful

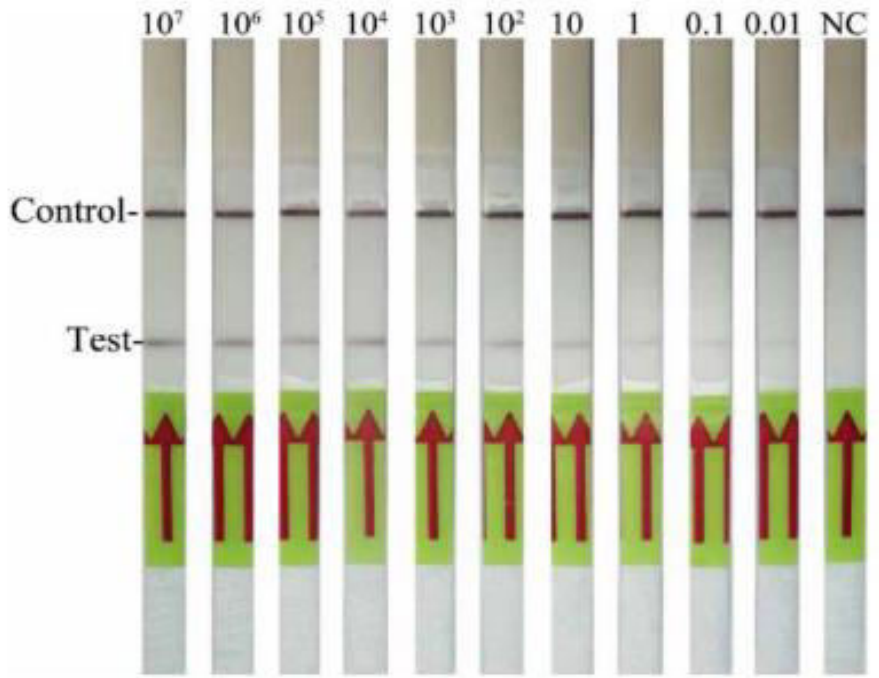


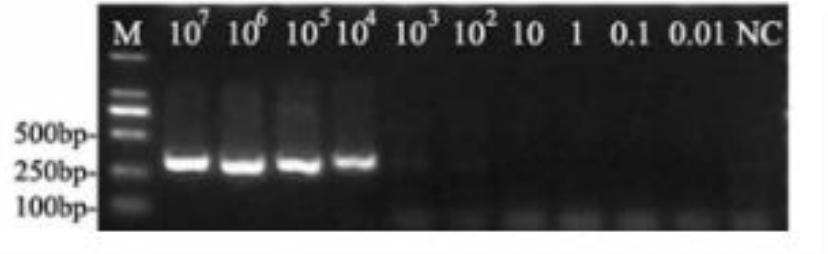


Assay	Primer Name	Sequence (5′ – 3′)
PCR	16S – 118F	ACTTGAGGAATCAGCCTCGG
	16S – 414R	TTTCGCTCCCCTAGCTTTCG
RPA	16S-RPA212F	TCAGCCAAGTCTGCCGTCAAATCAGGTTGC
	16S-RPA461R	AAGCCACGCCTAGTATCCATCGTTTACGGC
LF-RPA	16S-LF – 212F	TCAGCCAAGTCTGCCGTCAAATCAGGTTGC
	16S-LF – 461R	Biotin-AAGCCACGCCTAGTATCCATCGTTTACGGC
	16S-LF-P	FAM-CGGTGGAAACTGGCAGACTAGAGAGCAGTAGG-THF-GTAGCAGGAATTCCCAG- SpacerC3

- The **specificity** of LF-RPA was evaluated using the eight cyanobacterial species (lanes 1–8) and five non-cyanobacterial controls.
- PCR and LF-RPA were compared for their detection **sensitivity** using DNA samples from different starting cell concentrations of *Microcystis aeruginosa* cells.







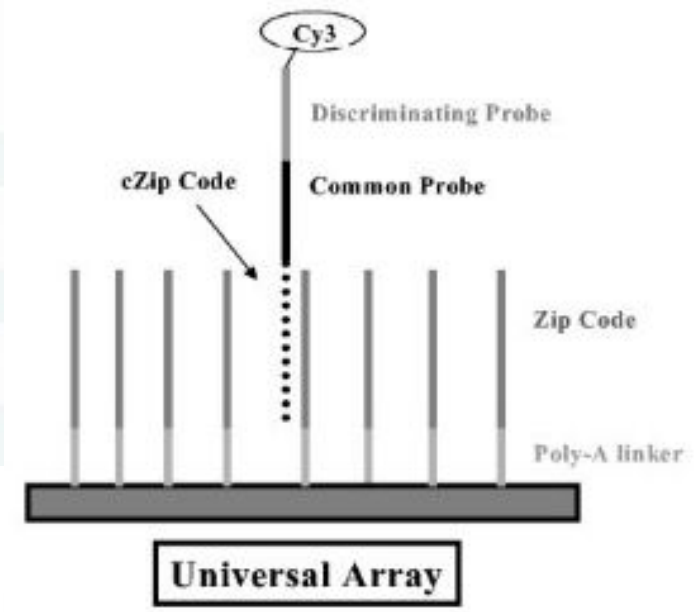
Castiglioni B, Rizzi E, Frosini A, *et al*. Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. *Appl Environ Microbiol*. 2004;70(12):7161-7172.

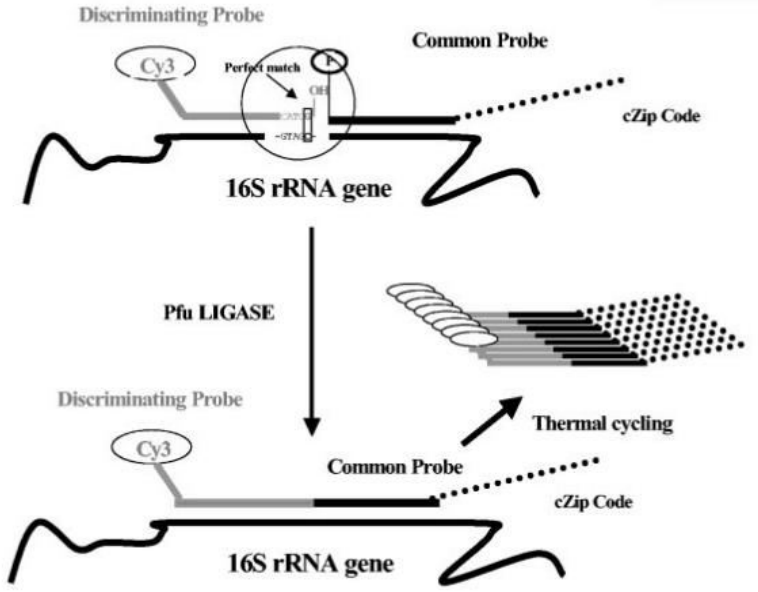
- Procedure based on the discriminative properties of the **DNA ligation detection reaction (LDR)**
- **16S rRNA** sequences of 19 different cyanobacterial groups were targeted
- Two probes specific for each target sequence required:

1) First carrying a **fluorescent dye**
2) Second a complementary **zip-code**

- Microarray validation:

- Axenic and nonaxenic strains of cyanobacteria
- Environmental sample



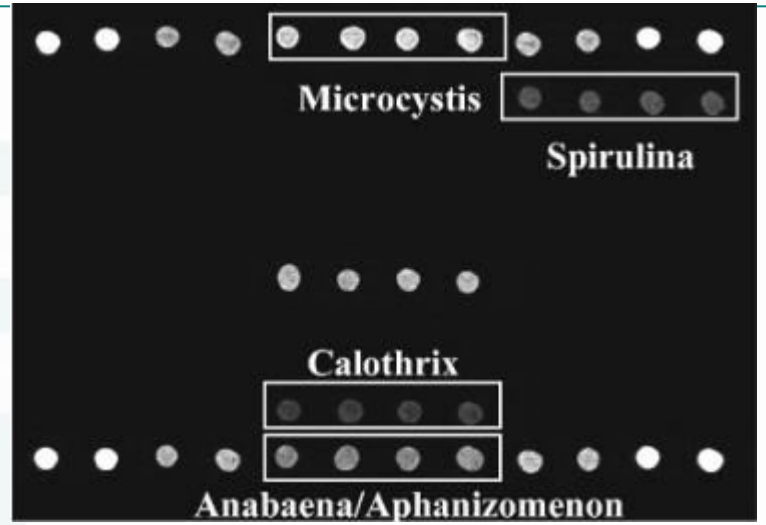


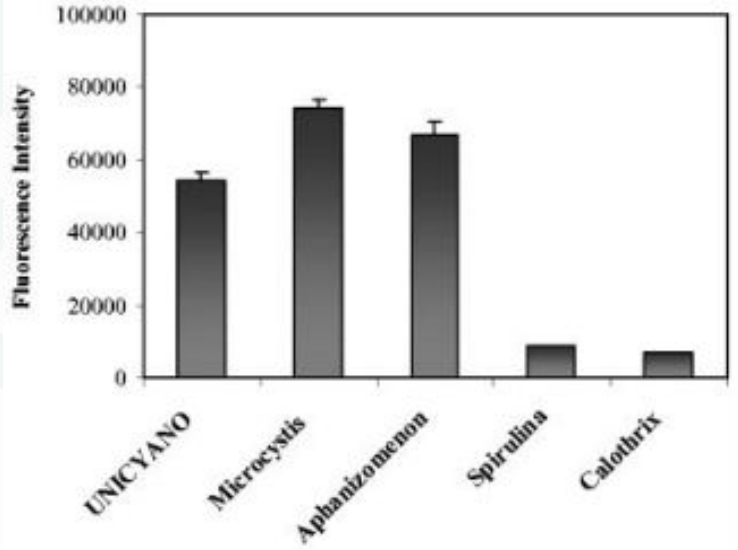
- Only group-specific spots, universal spots, and those spots corresponding to the hybridization control showed positive signals.

- The unbalanced LDR mix contained:
- 100 fmol of the PCR product from both *M. aeruginosa* strain PCC 9354 and *Aphanizomenon sp.* strain 202
- 5 fmol of the PCR product of both *Spirulina* strain PCC 6313 and *Calothrix* sp. strain PCC 7714.

- The array is termed universal, because these zip code pairs could be used with any other probe set.

"This technology can be easily applied to the future study of other marker genes, one of the most interesting of which would be the combination of the array developed here with one that could detect **potentially toxic cyanobacteria**. This would reveal the genetic diversity of cyanobacteria as well as the presence of potentially toxic genotypes in a sample."





The current analytical methods for the detection of microcystins in blooms:

https://www.niehs.nih.gov/health/assets/images/harmful_algal_blooms.jpg

1. HPLC,
2. PCR,
3. Mouse bioassays,
4. Protein phosphatase inhibition assays,
5. ELISA,
6. Mass spectrometry,
7. Raman spectroscopy.

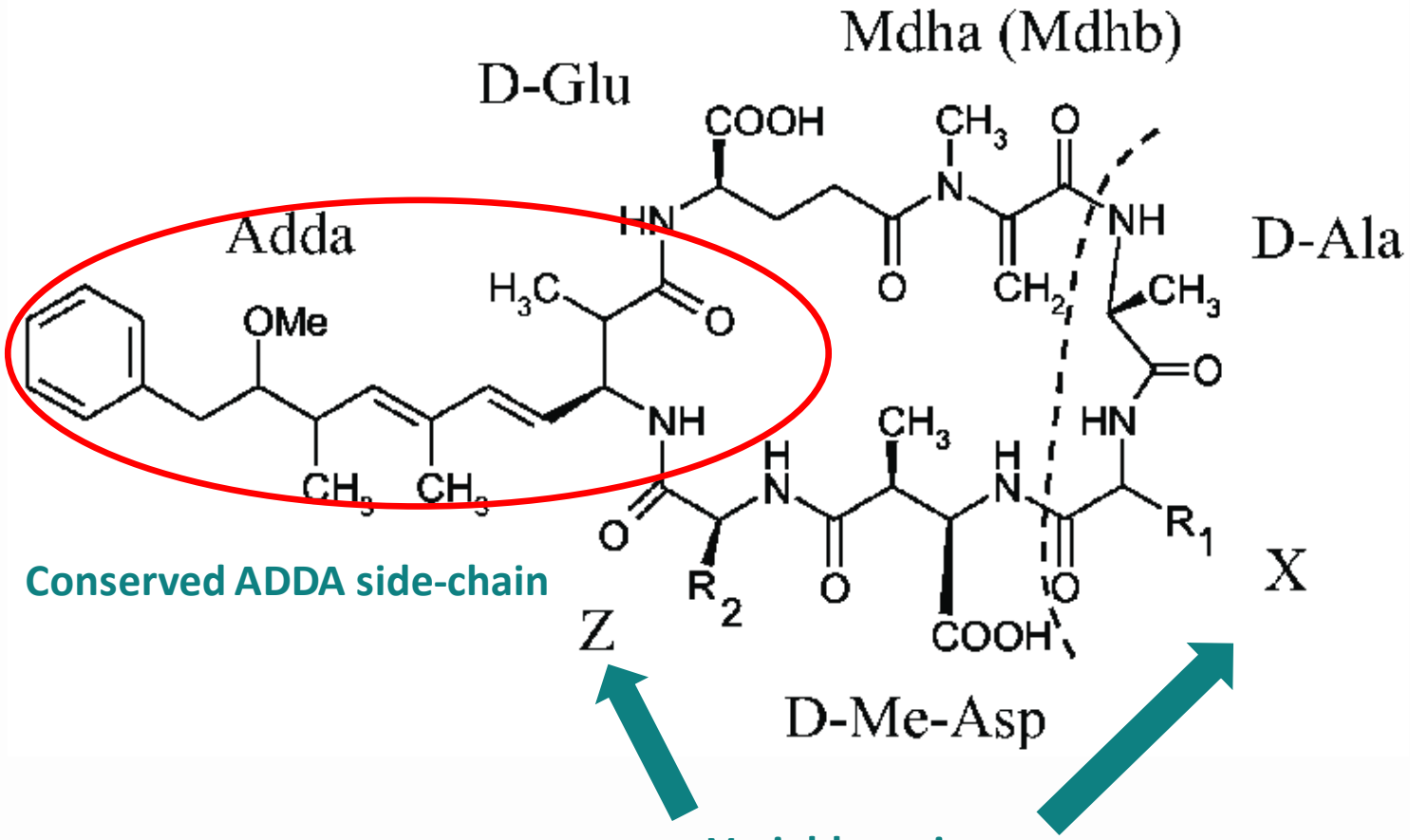
They all require specialized high-cost instruments and consumables!

Zhu, P., Zhang, B.-F., Wu, J.-H., Dang, C.-Y., Lv, Y.-T., Fan, J.-Z., & Yan, X.-J. (2014). *Sensitive and rapid detection of microcystin synthetase E Gene (mcyE) by loop-mediated isothermal amplification: A new assay for detecting the potential microcystin-producing Microcystis in the aquatic ecosystem. Harmful Algae, 37, 8–16.*

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

Microcystins - structure

https://www.researchgate.net/profile/Liang_Chen78/publication/281380183/figure/fig2/AS:296446600466435@1447689785434/Microcystins-MCs-in-the-aquatic-environment-The-cyanobacteria-compete-with-other.png

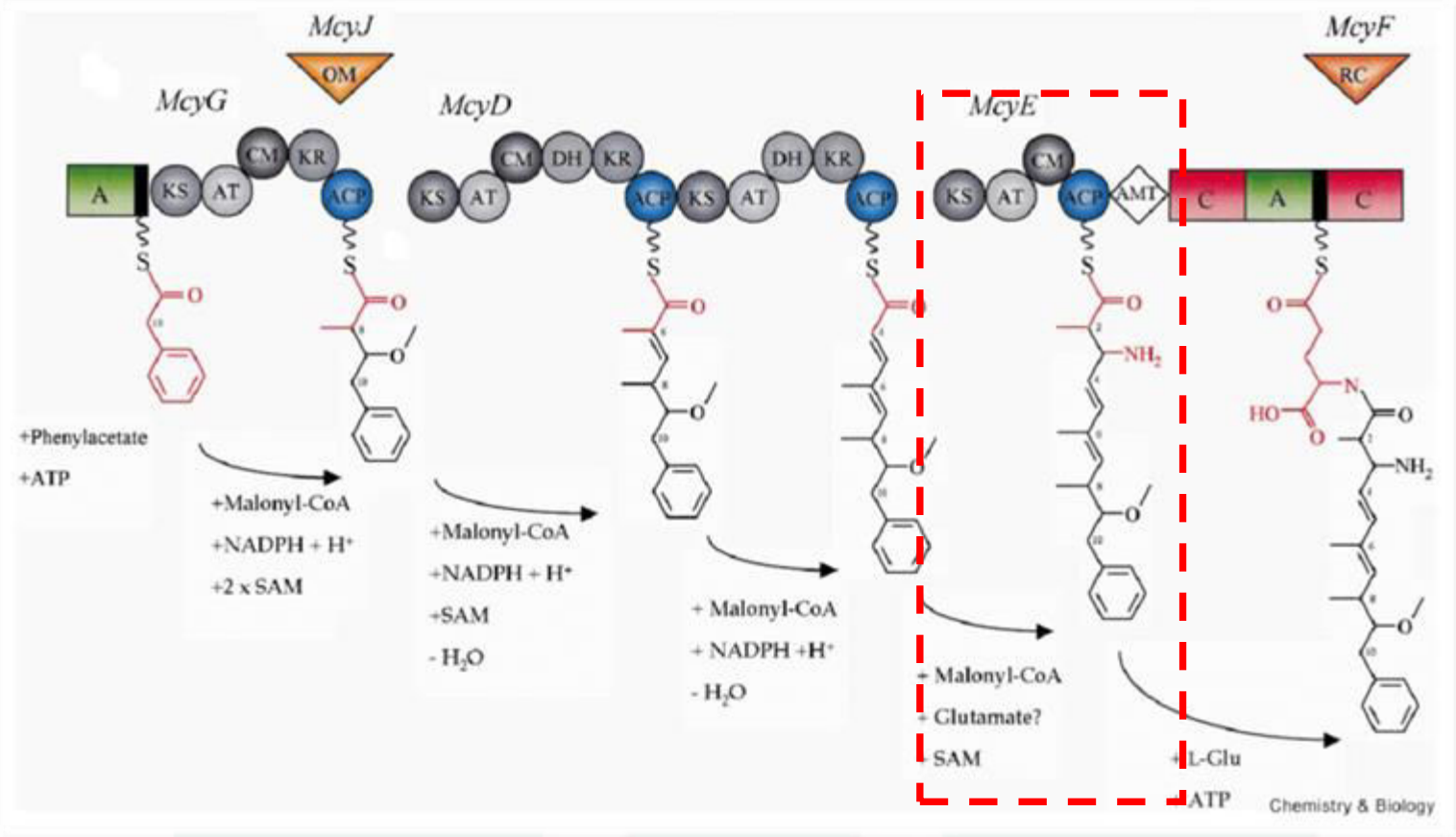


Type	X	Z
MC-LR	Leu (L)	Arg (R)
MC-RR	Arg (R)	Arg (R)
MC-YR	Tyr (Y)	Arg (R)
MC-LA	Leu (L)	Ala (A)

https://www.researchgate.net/profile/Anne_Ylinen2/publication/47932349/figure/fig4/AS:668901385973760@1536489926057/The-general-structure-of-microcystins-and-nodularin-Microcystins-contain-seven-amino.png

LAMP for detection of *mcyE* gene

- LAMP for detection of microcystin synthetase E Gene (*mcyE*) that encodes a protein (McyE) being responsible to catalyze the addition of D-glutamate to Adda.



https://www.researchgate.net/profile/Daniel_Tillett2/publication/12291472/figure/fig4/AS:667705925435397@1536204906446/Model-for-the-formation-of-Adda-and-predicted-domain-structure-of-McyG-McyD-and-McyE.png

Zhu, P., Zhang, B.-F., Wu, J.-H., Dang, C.-Y., Lv, Y.-T., Fan, J.-Z., & Yan, X.-J. (2014). ***Sensitive and rapid detection of microcystin synthetase E Gene (mcyE) by loop-mediated isothermal amplification: A new assay for detecting the potential microcystin-producing Microcystis in the aquatic ecosystem. Harmful Algae, 37, 8–16.***

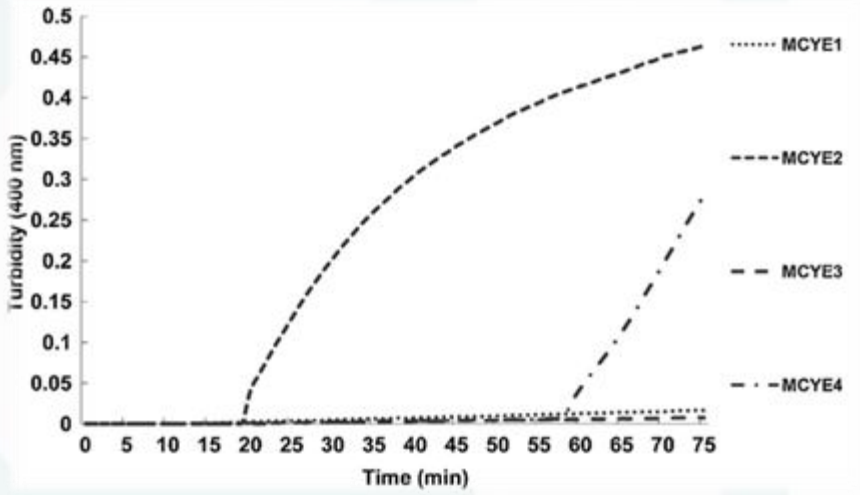
Primer design

PrimerExplorer V4
Please click for software information

- Four sets of primers were designed to recognize six distinct sequences on target the *mcyE* gene and applied on Microcystis aeruginosa FACHB-925 to establish the most appropriate primers of LAMP.

Table 2
Primers for LAMP amplification of microcystins.

Primer	Type	Length	Sequence (5′–3′)
MCYE1-F3	Forward-outer primer	18-mer	ATAACCCGATGCTAGGGC
MCYE1-B3	Backward-outer primer	21-mer	GCAATGTTTACGGATCTACAG
MCYE1-FIP	Forward-inner primer (F1c+TTTT+F2)	49-mer	CGCTTAGGGGTTCTTGATGAATTTA *TTTT* CCTTCTACACAAATTTCGCC
MCYE1-BIP	Backward-inner primer (B1c+TTTT+B2)	48-mer	TTGTTATGGAAAACTTGACCGAGG *TTTT* AAACTTGTCTGGCAGTATCC
MCYE2-F3	Forward-outer primer	25-mer	GGAACGGGATTATTAAATTCATCAA
MCYE2-B3	Backward-outer primer	24-mer	CTTAAAATATATCTCGGTAGGAGG
MCYE2-FIP	Forward-inner primer (F1c+TTTT+F2)	47-mer	TGGGAGTATCCCAGTATGAAATCAA *TTTT* CGATTGTTATGGAAAACTTGAC
MCYE2-BIP	Backward-inner primer (B1c+TTTT+B2)	44-mer	ACAAGTTTCTGTAGATCCGTAAACA *TTTT* AGATCGGGAAACTGCCTTA
MCYE2-LF	Loop-forward primer	18-mer	ACAGCGAAATCCCCCTCG
MCYE2-LB	Loop-backward primer	21-mer	TTCTGCTGGAAAACGTTTCCG
MCYE3-F3	Forward-outer primer	18-mer	GTGGTGTGTAGGGCATTA
MCYE3-B3	Backward-outer primer	21-mer	ATTCAAAGCTTACGACTTACC
MCYE3-FIP	Forward-inner primer (F1c+TTTT+F2)	52-mer	ACCATAGGTGCATCCCTATATATGT *TTTT* TTCCTGTAGGAAAGAAACCAAAT
MCYE3-BIP	Backward-inner primer (B1c+TTTT+B2)	52-mer	GATGGATCATGAGTAACAGCAGC*TTTT* TGATTTAGATAAACATCCTGAATGG
MCYE4-F3	Forward-outer primer	23-mer	TTTTCTCCATTTTTTCAGGTAAC
MCYE4-B3	Backward-outer primer	18-mer	GTCATGCTCCAGCGTAAT
MCYE4-FIP	Forward-inner primer (F1c+TTTT+F2)	51-mer	CTCACAGTCGCATTGAGTTTATTTT *TTTT* TAAAATTACTCTCCGTTAGGCA
MCYE4-BIP	Backward-inner primer (B1c+TTTT+B2)	51-mer	TGAGAGTCTATAGGAACGTAAGTCG *TTTT* CACTACTTAATCATCGGGATTC



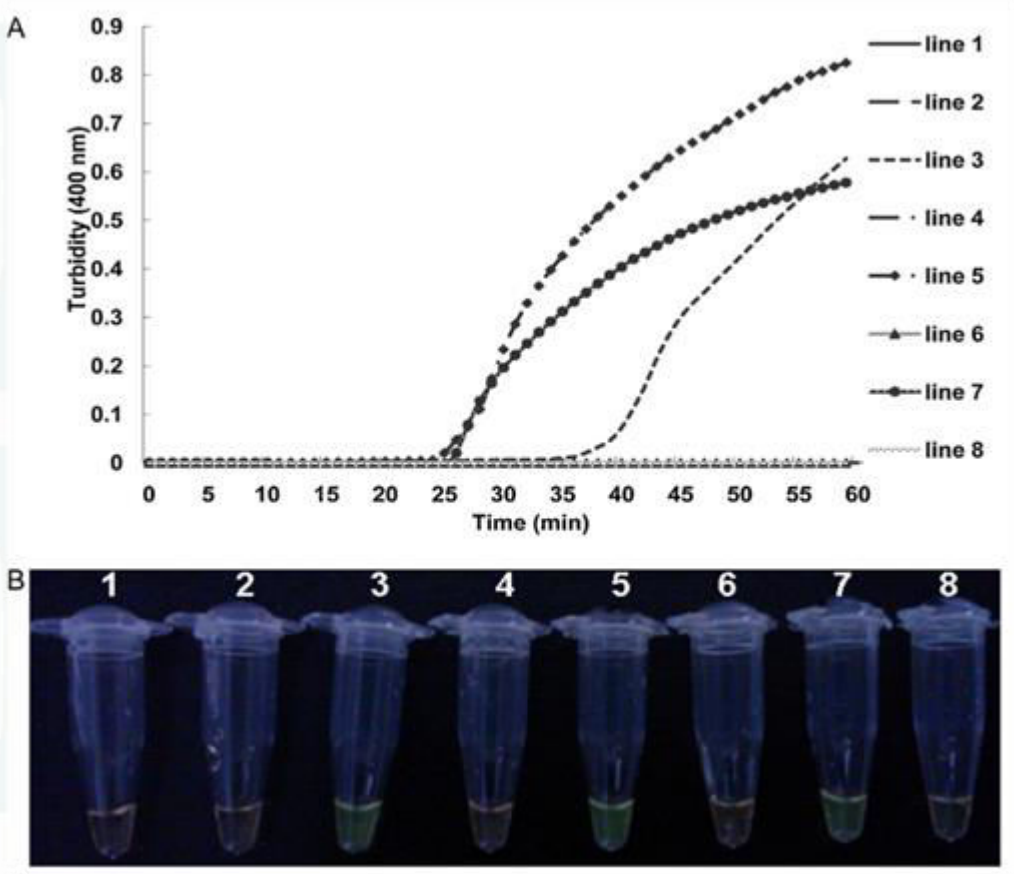
The primers in MCYE2 primer set were chosen as the final optimal primers for *mcyE* detection by LAMP

Zhu, P., Zhang, B.-F., Wu, J.-H., Dang, C.-Y., Lv, Y.-T., Fan, J.-Z., & Yan, X.-J. (2014). *Sensitive and rapid detection of microcystin synthetase E Gene (mcyE) by loop-mediated isothermal amplification: A new assay for detecting the potential microcystin-producing Microcystis in the aquatic ecosystem. Harmful Algae, 37, 8–16.*

Analysis of environmental water samples

- The microcystin-producing gene and microcystins were synchronously analyzed using the **LAMP and ELISA** assay.
- ELISA → one microcystin-positive sample concentration is approximately 0.63 mg/l.
- LAMP → the *mcyE* gene was detected in three water samples.

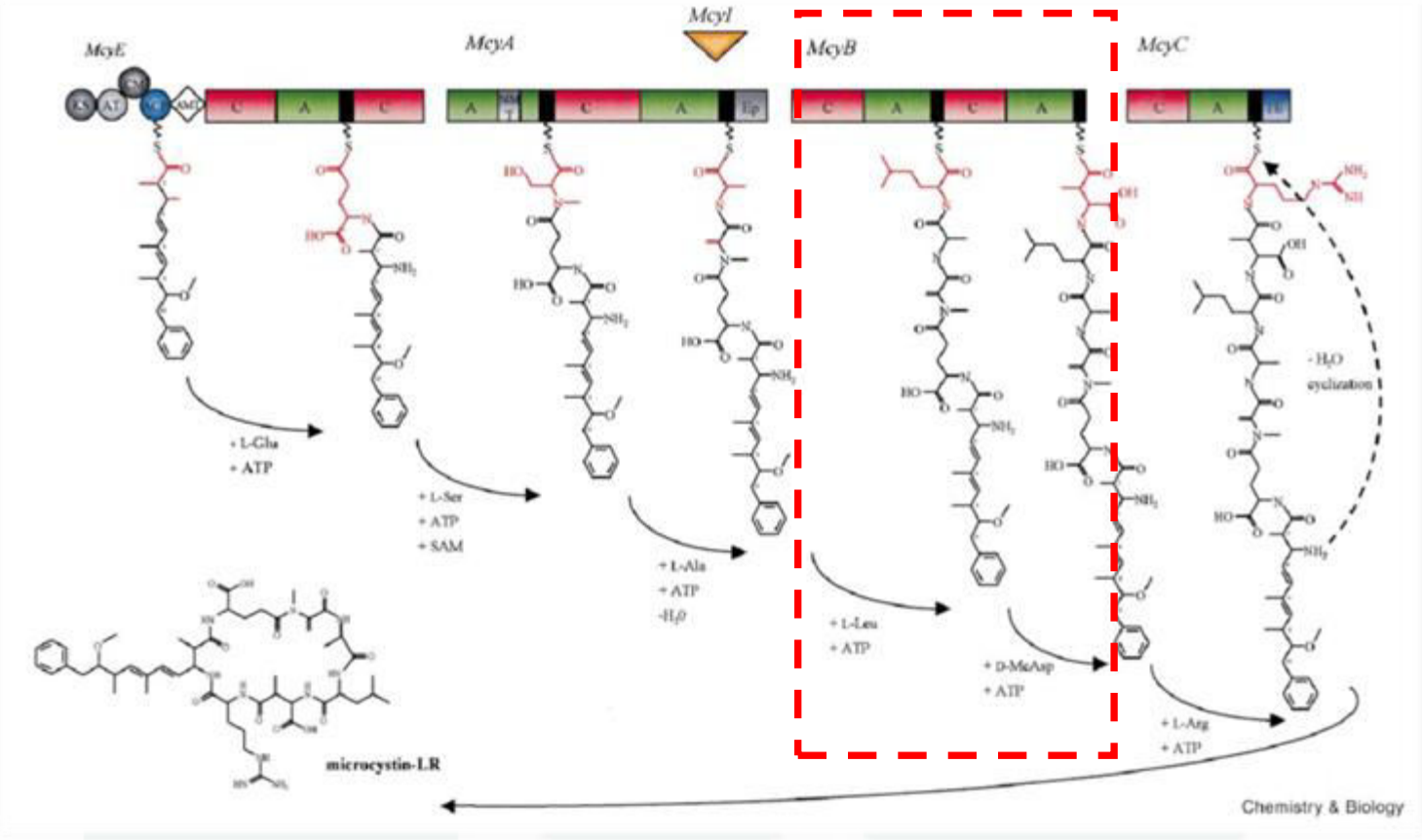
The positive results of LAMP assay suggested the concentration of microcystin in two samples were below the ELISA detection limit, but microcystin could be in risk during bloom in these two places



Zhu, P., Zhang, B.-F., Wu, J.-H., Dang, C.-Y., Lv, Y.-T., Fan, J.-Z., & Yan, X.-J. (2014). *Sensitive and rapid detection of microcystin synthetase E Gene (mcyE) by loop-mediated isothermal amplification: A new assay for detecting the potential microcystin-producing Microcystis in the aquatic ecosystem. Harmful Algae, 37, 8–16.*

LAMP for detection of *mcyB* gene

- The *mcyB* gene is known to be involved in the addition of L-Leu and D-MeAsp to the growing polypeptide chain of microcystin biosynthesis.
- Since *mcyB* gene plays an important role in the formation of microcystin, detecting the microcystin-producing species using the gene as a marker could be more sensitive and reliable.



https://www.researchgate.net/profile/Daniel_Tillett2/publication/12291472/figure/fig5/AS:667705925451779@1536204906493/Biosynthetic-model-for-microcystin-LR-and-predicted-domain-structure-of-McyE-McyA-McyB_W640.jpg

Ramya, M., Kayalvizhi, M., Haripriya, G., & Rathinasabapathi, P. (2018). ***Detection of microcystin-producing cyanobacteria in water samples using loop-mediated isothermal amplification targeting mcyB gene. 3 Biotech, 8(9).***

Experimental main steps

1. The genomic DNA from *M. aeruginosa* (NIES-843) was isolated using the CTAB method.
2. The *mcyB* gene was amplified using PCR with the gene-specific primers:

   FP: 5′ CCC CAG GCA AGC AGA AAT TCA GGA 3′
   RP: 5′ CCC CAT AGC AAC CACC GTCAA AGG 3′

3. The amplified *mcyB* gene was cloned into a pGEM-T Easy Vector (Promega, USA), and transformed into *Escherichia coli* Top10 strain (Invitrogen).
4. The positive clones were selected and the plasmids harboring the *mcyB* gene (pMCYB) were isolated and subsequently used for both PCR and LAMP assays.

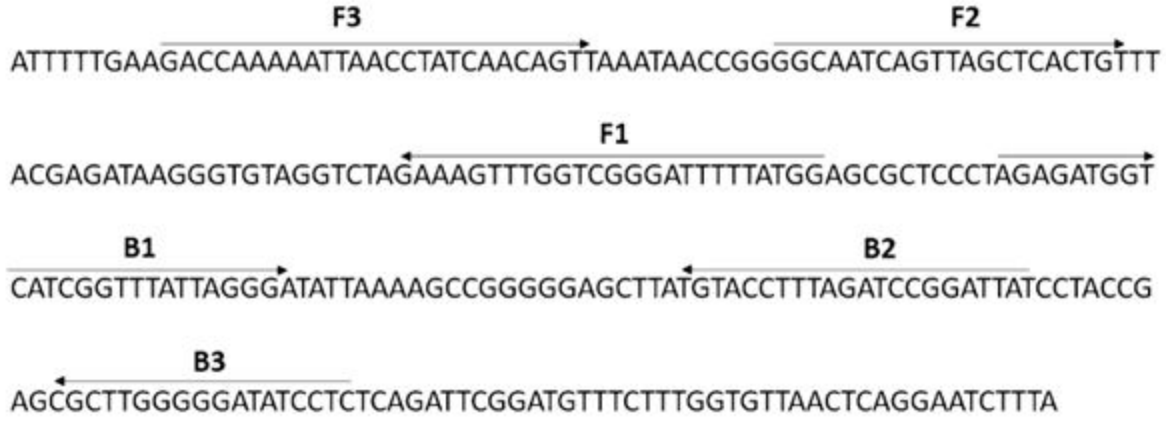
Ramya, M., Kayalvizhi, M., Haripriya, G., & Rathinasabapathi, P. (2018). ***Detection of microcystin-producing cyanobacteria in water samples using loop-mediated isothermal amplification targeting mcyB gene. 3 Biotech, 8(9).***

Primer design

PrimerExplorer V4
Please click for software information

- The LAMP primers recognize six distinct regions in the *mcyB* gene

A



B

Primer	Sequence (5' to 3')
FIP	CCATAAAAATCCCGACCAAACTTTCTTTTGGCAATCAGTTAGCTCACTG
BIP	GAGATGGTCATCGGTTTATTAGGGTTTTATAATCCGGATCTAAAGGTACA
F3	GACCAAAAATTAACCTATCAACAGT
B3	AGAGGATATCCCCCAAGC

A - Location and sequence of LAMP targets and priming sites for the *mcyB* gene.

B - Sequences of LAMP primers.
Primer FIP consisted of the F1 complementary sequence and F2 direct sequence. Primer BIP consisted of B1 direct sequence and B2 complementary sequence.

Ramya, M., Kayalvizhi, M., Haripriya, G., & Rathinasabapathi, P. (2018). ***Detection of microcystin-producing cyanobacteria in water samples using loop-mediated isothermal amplification targeting mcyB gene. 3 Biotech, 8(9).***

Detection of microcystin-producing cyanobacteria in water samples

- The 21 concentrated water samples were directly used for PCR and LAMP analysis.

Table 1 Screening of environmental and drinking water samples for the presence of toxic cyanobacteria

Samples	No of sample screened	PCR+	LAMP+
Open lakes and ponds	9	4	5
Water samples from bird sanctuary	2	2	2
Chlorinated drinking water sample	10	–	–

PCR+ Number of water sample shown positive amplification by PCR, *LAMP+* Number of water sample shown positive for *mcyb* gene by the LAMP

We can get 1000-fold higher sensitivity of LAMP than PCR in detecting the *mcyB* gene.

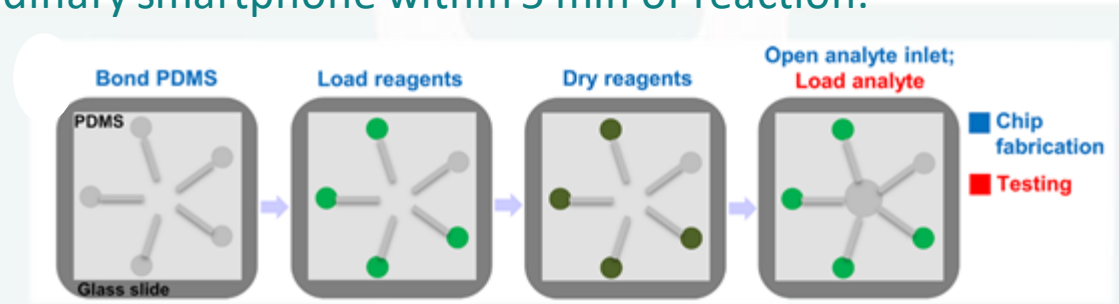
Hence, the developed method could be used for toxic cyanobacteria detection even before bloom formation.

Ramya, M., Kayalvizhi, M., Haripriya, G., & Rathinasabapathi, P. (2018). *Detection of microcystin-producing cyanobacteria in water samples using loop-mediated isothermal amplification targeting mcyB gene. 3 Biotech, 8(9).*

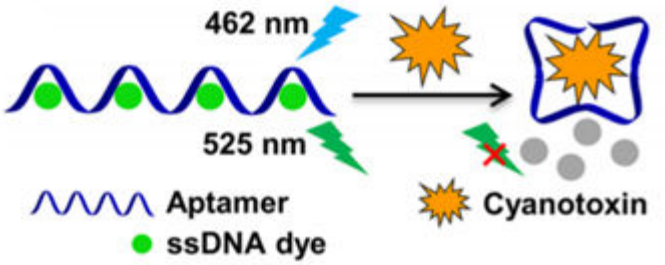
Li, Z., Zhang, S., Yu, T., Dai, Z., & Wei, Q. (2019). ***An Aptamer-Based Fluorescent Sensor Array for Rapid Detection of Cyanotoxins on a Smartphone***. *Analytical Chemistry*.

→ Differential fluorescent sensor array made of aptamers and single-stranded DNA (ssDNA) dyes for multiplexed detection and discrimination of four common cyanotoxins with an ordinary smartphone within 5 min of reaction.

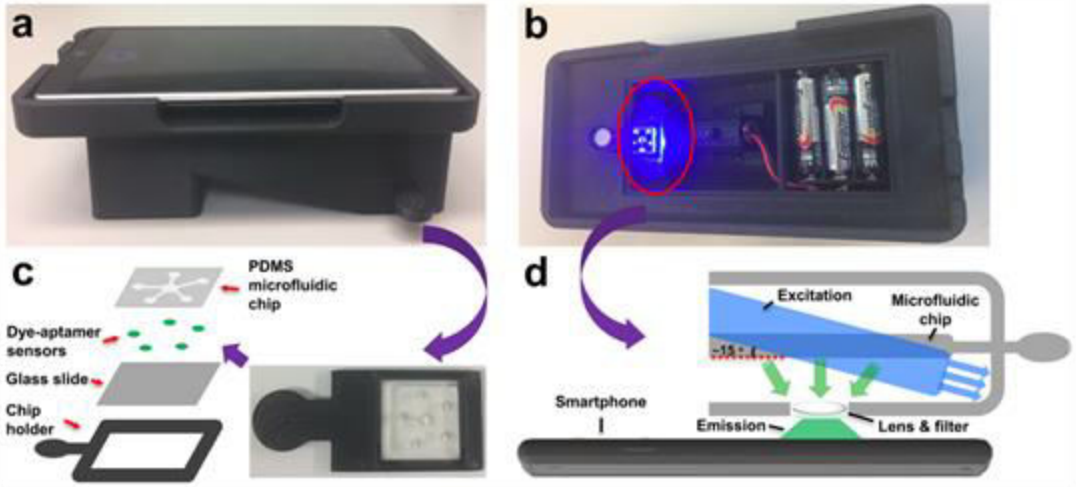
The assay reagents were preloaded and dried in a microfluidic chip with a long shelf life over 60 days.



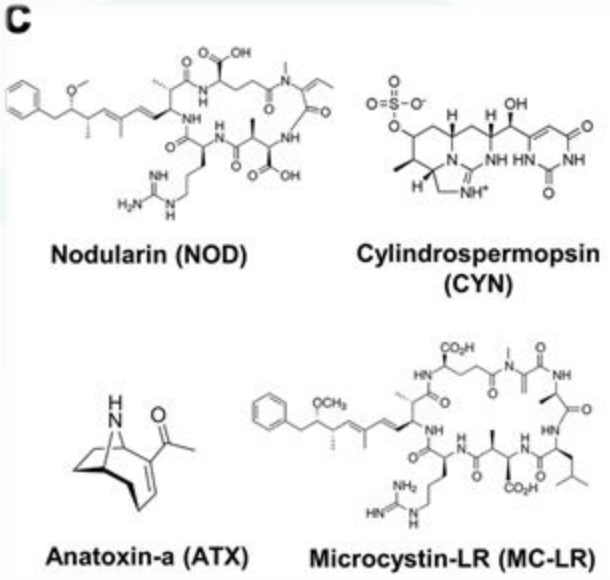
Upon the addition of analyte solutions, competitive binding of cyanotoxin to the specific aptamer-dye conjugate occurred



3D printed



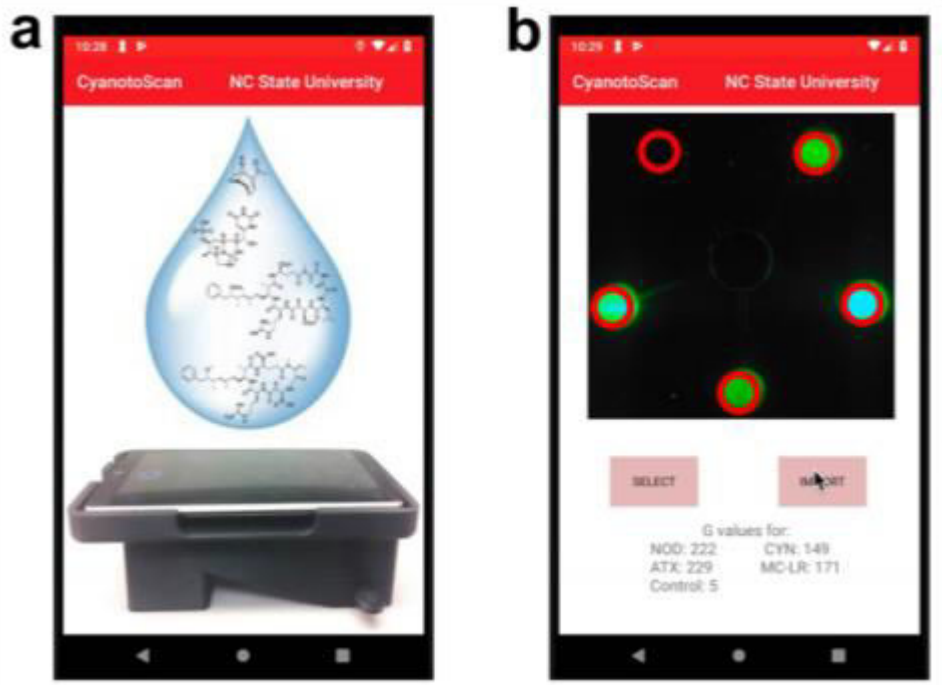
•**The cost of the entire attachment device is estimated to be ~$70, excluding the smartphone and emission filte.**



Li, Z., Zhang, S., Yu, T., Dai, Z., & Wei, Q. (2019). ***An Aptamer-Based Fluorescent Sensor Array for Rapid Detection of Cyanotoxins on a Smartphone***. *Analytical Chemistry*.

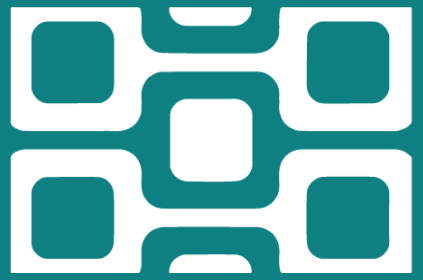
Target	Aptamer sequence	Calculated K_d (nM)	Reported K_d (nM)	Ref
ATX	5'-TGG CGA CAA GAA GAC GTA CAA ACA CGC ACC AGG CCG GAG TGG AGT ATT CTG AGG TCG G -3'	76	81	1
CYN	5'-CAC AGC GTG ACA CGG CAA CCC ACC CAC CAG CCC CGT CAA CGA CCT ATA CCT GTG CTG CGC A-3'	87	87	2
NOD	5'-AAG GAG CAG CGT GGA GGA TAG TCG CGG CCG GGT TCG GCA GGA ATG GGG CGT TGT TTG GGG TTA GGG TGT GTC GTC GTG GT-3'	123	139	3
MC-LR	5'-GGC GCC AAA CAG GAC CAC CAT GAC AAT TAC CCA TAC CAC CTC ATT ATG CCC CAT CTC CGC-3'	87	50	4

Sample	Spiked (nM)				Detected (nM)				Recovery Rates (%) & RSD (%)			
	ATX	CYN	NOD	MC-LR	ATX	CYN	NOD	MC-LR	ATX	CYN	NOD	MC-LR
1	15.0				15.7				104.6 & 1.7			
2		18.0				17.3				97.1 & 1.2		
3			24.0				24.4				101.7 & 2.1	
4		17.0		10.0		17.8		9.7		102.9 & 2.4		97.0 & 1.6
5	18.0	24.0	30.0	15.0	17.7	23.7	29.3	15.4	98.3 & 1.6	98.8 & 2.5	97.7 & 0.9	102.7 & 2.8



Screenshots of the smartphone app.

Measurement results of five lake water samples spiked with varied types and concentrations of four cyanotoxins.



IPANEMA

Thank you for your attention!